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Exploration of inflammasomes as targets for therapy of Pseudomonas aeruginosa infection

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Exploration of Inflammasomes as Targets for Therapy of *Pseudomonas aeruginosa* Infection

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Abstract

Pseudomonas aeruginosa is a common Gram-negative opportunistic bacterial pathogen capable of infecting humans with compromised natural defenses and causing severe pulmonary disease. It is the major cause of severe chronic pulmonary disease in cystic fibrosis (CF) patients subsequently resulting in progressive deterioration of lung function. Interaction between *P. aeruginosa* and host induces a number of marked inflammatory responses and is associated with complex therapeutic problems. NOD-like receptors (NLRs) can recognize a variety of endogenous and exogenous ligands and its activation initiate inflammasome formation that induces maturation of the pro-inflammatory cytokine interleukin (IL)-1β through activation of caspase-1. Through a literature search, no prior research on mutant strains as well as clinical isolates of *P. aeruginosa* from CF patients at different stages of infection has been conducted to explore NLR-mediated innate immune responses to this bacterial infection. All the work presented in this thesis focuses on the exploration of inflammasomes as targets for therapy of *P. aeruginosa* infection. We hypothesized that genetic alterations of *P. aeruginosa* affect the innate immune response of human monocytes. THP-1 human monocytic cells were infected with clinical *P. aeruginosa* isolates from CF patients, or with *P. aeruginosa* mutant strains lacking flagella, pili, lipopolysaccharide, or pyocyanin. The overall involvement of NLRs in innate immune recognition of *P. aeruginosa* was addressed through demonstrating of NLR-mediated caspase-1 activation or *P. aeruginosa*-induced IL-1β secretion. Our findings suggest that *P. aeruginosa*, which lost certain virulence factors during pulmonary infection, may fail to induce caspase-1

activation and secretion of IL-1β in the process of host-pathogen interactions. This may reveal novel mechanism of the pathogen adaptation to avoid detection by NLR(s).

As *P. aeruginosa* infections are characterized by strong inflammation of infected tissues anti-inflammatory therapies in combination with antibiotics have been considered for the treatment of associated diseases. Spleen tyrosine kinase (SYK), a non-receptor tyrosine kinase, is an important regulator of inflammatory responses. Several studies have highlighted SYK as a key player in the pathogenesis of a multitude of diseases. Inhibition of SYK activity was explored as a therapeutic option in several inflammatory conditions; however, this has not been studied in bacterial infections. We used a model of an *in vitro* infection of human monocytic cell line THP-1 and lung epithelial cell line H292 with both wild type and flagella-deficient mutant of *P. aeruginosa* strain K, as well as with clinical isolates from CF patients, to study the effect of a small molecule SYK inhibitor R406 on inflammatory responses induced by this pathogen. The role of SYK in regulation of inflammasome activation was also determined by evaluating the effect of SYK inhibitor on innate immune responses in *P. aeruginosa* infected cells. The results suggest that SYK is involved in the regulation of inflammatory responses to *P. aeruginosa*, and R406 may potentially be useful in dampening the damage caused by severe inflammation associated with this infection.

Keywords: *P. aeruginosa*, Cystic Fibrosis, Innate immunity, host-pathogen interaction, NOD-like receptors, Caspase-1, Cytokines, SYK, small molecule inhibitor, R406

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List of Abbreviations

°C	Celsius degree
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
g	Gram or relative centrifugal force (RCF)
h	Hour(s)
kDa	Kilo Dalton
L	liter(s)
LB	Luria-Bertani
M	Molar(s)
mg	Milligram(s)
min	Minute(s)
mL	millilitre(s)
mM	millimolar(s)
MOI	Multiplicity of infection
PBS	Phosphate buffered saline
pH	Negative logarithm of hydrogen ion concentration
RPMI	Roswell Park Memorial medium
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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To my parents

My mother Amnah Alfageh & My father Ali Alhazmi

Whose affection, love, encouragement, and prays of day and night make me able to get such success and honor

To my sisters and brothers

Ms. Najla Alhazmi, Ms. Najwa Alhazmi, Ms. Nehad Alhazmi,

&

Mr. Adel Alhazmi, Mr. Adnan Alhazmi, & Mr. Saud Alhazmi

For their advice, patience, and faith

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For supporting me all the way

Co-Authorship Statement

The following thesis is composed of three published manuscripts and two manuscripts awaiting publication.

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Chapter I: Literature Review

1.1 *Pseudomonas aeruginosa* – Pathogenesis and Pathogenic Mechanisms

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Abstract

Pseudomonas aeruginosa is a common Gram-negative opportunistic bacterial pathogen capable of infecting humans with compromised natural defenses and causing severe pulmonary disease. It is one of the leading pathogens associated with nosocomial infections. It has a vast arsenal of virulence factors that are used to interfere with host defenses. *P. aeruginosa* virulence factors facilitate adhesion, modulate or disrupt host cell signaling pathways, and target the extracellular matrix. The propensity of *P. aeruginosa* to form biofilms further protects it from antibiotics and the host immune system. *P. aeruginosa* is intrinsically resistant to a large number of antibiotics and can acquire resistance to many others, making treatment difficult. *P. aeruginosa* provokes a potent inflammatory response during the infection process. Most deaths in immunocompromised patients with cystic fibrosis is attributed to the progressive decline of lung function resulting from chronic infection caused by *P. aeruginosa*. Antibiotic treatment of chronic *P. aeruginosa* infections may temporarily suppress symptoms; however, this does not eradicate the pathogen. Lung diseases caused by *P. aeruginosa* are a leading cause of death in immunocompromised individuals. Although leukocyte recruitment is critical for the host defense, excessive neutrophil accumulation results in life-threatening conditions,

such as acute lung injury. Several *P. aeruginosa* virulence factors have been studied as potential vaccine candidates, although there is currently no clinically accepted vaccine. Understanding host-pathogen interactions is critical for the development of effective therapeutic strategies to control the damage in the lung caused by this infection.

Keywords: *Pseudomonas aeruginosa*, nosocomial infection, virulence factors, antibiotic resistance, cystic fibrosis

P. aeruginosa is a motile, non-fermenting, Gram-negative organism belonging to the family Pseudomonadaceae. In 1850s, Sédillot observed that a blue-green discharge was frequently present and associated with infection in surgical wound dressings [1]. The infectious organism was a rod-shaped and blue-green pigmented bacterium [2]. By 1961, the ability of this organism to cause both severe acute and chronic infections was recognized [3]. In 1960s, *P. aeruginosa* emerged as an important human pathogen [4]. Despite anti-pseudomonas activity being one of the pharmaceutical drug discoveries for several decades, it remains one of the most recalcitrant and difficult to treat organisms. Accordingly, *P. aeruginosa* is considered as a Superbug.

Genome analysis revealed that *P. aeruginosa* genome has a markedly large genome (6.3 million base pair (Mbp); encoding 5567 genes) compared to 4.64 Mbp (4279 genes) in *Escherichia coli* K12, 2.81 Mbp (2594 genes) in *Staphylococcus aureus* N315, and 1.83 Mbp (1714 genes) in *Haemophilus influenzae* Rd. Also, the proportion of predicted regulatory genes in *P. aeruginosa* genome is greater than in all other sequenced bacterial genomes [5,6], lending to its adaptability to various environments. *P. aeruginosa* has a broad range of growth substrate, minimal nutrient requirements and it is a non-fastidious

microorganism [7]. The organism is tolerant of temperatures as high as 50ºC and is capable of growing under aerobic, as well as anaerobic conditions [8]. Due to possessing a large number of virulence factors *P. aeruginosa* is truly a challenging pathogen in the hospital setting as it is resistant to many antibiotics. Also, it is capable of forming highly resistant biofilms, both within the body and on the surfaces of medical instruments [9-11]. *P. aeruginosa* continues to be problematic from a treatment perspective.

P. aeruginosa is armed with potent virulence factors. Although ubiquitously present in the environment, *P. aeruginosa* never causes disease in an immunocompetent host as the immune system effectively prevents the infection. However, the pathogen causes severe infections in Cystic Fibrosis (CF) patients. In CF, a genetic defect in cystic fibrosis transmembrane conductance regulator (CFTR) underlies the development of persistent infection with *P. aeruginosa* that gradually leads to irreversible tissue damage. Several conserved microbial structures in *P. aeruginosa* are recognized by Toll-like receptors (TLRs) and NOD-like receptors (NLRs); which have been implicated in activating the host innate immune responses to *P. aeruginosa* [12,13]. There are a number of clinical conditions associated with *P. aeruginosa* infection. *P. aeruginosa* is an opportunistic organism infecting burn victims, CF, leukemic, transplant, neutropenic, long-term urinary catheters, and diabetic patients as well intravenous drug abusers.

1.1.1. Nosocomial Infection Due to *P. aeruginosa*

Nosocomial (hospital-acquired) infections are those not present at the time of hospital admission, but they usually develop post-admission. The 2006-2007 report by the National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention (CDC) ranked *P. aeruginosa* as the sixth most common pathogen associated

with healthcare associated infections. The NHSN reports that in United States in 2006-7, 8% of all hospital-associated infections were due to *P. aeruginosa*, with *P. aeruginosa* causing 3% of central line-associated bloodstream infections, 6% of surgical site infections, 10% of catheter-associated urinary tract infection and 16% of ventilator-associated pneumonia (VAP) infections [14]. Mechanical ventilation, antibiotic therapy, surgery, and chemotherapy are the major predisposing factors contributing to the acquisition of a *P. aeruginosa* infection in the hospital [15]. It is worth noting however, that difficulties in treatment of such infections and the associated morbidity and mortality, have made *P. aeruginosa* one of the most feared hospital pathogens.

1.1.1.1 Burn Wound Infections

P. aeruginosa is the leading cause of invasive infections in burn patients; 75% of all deaths in patients with severe burns are related to sepsis from invasive burn wound infection [16-18]. In addition to skin injury, inhalation injury is common in burn patients. This results in edema and sloughing of the respiratory tract mucosa and impairment of the normal mucociliary clearance mechanism, thus making these patients more susceptible to upper respiratory tract infections as well as *P. aeruginosa* pneumonia [19].

Gram-positive organisms such as *Staphylococcus aureus* and *Streptococcus pyrogens* are typically the first microorganisms to colonize the site of infection, following by other microbes including *P. aeruginosa* colonize these wounds [20,21]. Success with early wound excision practices was shown to contribute to the prevention of invasive infections disseminating from the wound site [22]. Animal studies of partial-thickness cutaneous burns showed that mature *P. aeruginosa* biofilms could develop during 48 to

72 hours, indicating a major potential source of further difficulties in antimicrobial therapy at these sites [23].

In addition to the *P. aeruginosa* virulence factors that undoubtedly contribute to the success of *P. aeruginosa* as a pathogen in the burn patients, the impairment of host immunity, beyond a simple loss of the skin's physical barrier, plays a role in enhancing susceptibility to infection. Recent studies have demonstrated that thermal injury causes impaired production of the host defense peptides β-defensins in the tissues surrounding the wound. These antimicrobial peptides have been proposed to play an important role in primary defense against *P. aeruginosa* and synthetic β-defensin was recently shown to be protective against *P. aeruginosa* infection in a burned mouse model [24].

1.1.1.2 Bacteremia

P. aeruginosa is among the five leading causes of nosocomial bacteremia and frequently leads to sepsis. In the 1960s and early 1970s, aminoglycosides and polymyxins were the only options for treatment of *P. aeruginosa* bacteremia but were found to be fairly ineffective for these infections. Mortality of greater than 50% was reported when mortality was used as the end point [25,26], and was as high as 70% in febrile neutropenic patients [27]. Despite the introduction of effective anti-pseudomonal β-lactams and the associated reduction in mortality rates, *P. aeruginosa* bacteremia is still one of the most feared nosocomial infections. These infections are generally associated with higher mortality compared to other infecting pathogens, and their persistence, particularly related to device-related bacteremia, continues to plague patients [28].

The main distinguishing feature of *P. aeruginosa* sepsis is the presence of ecthyma gangrenous, and these infarcted skin lesions occur only in markedly neutropenic patients

[29]. When *P. aeruginosa* disseminates from a site of local infection, it gains access to the bloodstream by breaking down epithelial and endothelial tissue barriers [30]. To evade the bactericidal activity of the serum complement, *P. aeruginosa* produces a smooth lipopolysaccharide (LPS) [31,32]; full-length O side-chain of the bacteria.

1.1.1.3 Hospital-Associated Pneumonia

The human respiratory tract presents a favorable environment to which *P. aeruginosa* has become particularly well adapted. *P. aeruginosa* has the formidable ability to cause both chronic infections in the lung of CF patients and acute nosocomial pneumonia [33]. Animal model studies of *P. aeruginosa* pneumonia have demonstrated the involvement of proteases, flagella, pili and LPS O side chains as well as the delivery of the extracellular toxins ExoS, ExoT and ExoU via a type III secretion system (T3SS) in the disease pathogenesis. For example, administration of anti-pcrV antibodies blocking the T3SS has been shown to offer protection against acute *P. aeruginosa* pneumonia when tested in animal models [34,35].

1.1.1.4 Ventilator-Associated Pneumonia

P. aeruginosa is commonly found to be the first or second major pathogen causing VAP [14]. It is the most common multidrug resistant pathogen involved in this disease and recovery rate of *P. aeruginosa* is increased with increased duration of mechanical ventilation. In addition to being amongst the most common pathogens causing VAP, *P. aeruginosa* is also amongst the most lethal pathogens, since reports suggest up to 70-80% mortality when the organism remains confined to the lung [36], with directly attributable mortality rates reaching 38% [37].

1.1.2. *P. aeruginosa* Infections in Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal reccesive genetic disorder and is the most common fatal genetic disease in the Caucasian population. CF is caused by a mutation in a gene on chromosome 7 known as CFTR. The most common mutation is ΔF508 (or F508del), which is a three-nucleotide deletion of a phenylalanine residue with subsequent defective intracellular processing of the CFTR protein, which is an important chloride channel [38]. CF is affecting 1:2,500 in the Caucasian population [39]. CF is multi-system disease, which affects mainly the lung and digestive system. Most CF-related deaths are due to lung disease [38].

Mortality in this afflicted population is mainly attributed to chronic respiratory infections and the associated gradual deterioration of lung function. There are several pathogens known to play a role in CF lung infection, with *Staphylococcus aureus* and *Haemophilus influenzae* being the predominant pathogens colonizing in infancy and early childhood, and eventual replacement by *P. aeruginosa*. However, *P. aeruginosa* is often isolated from patients less than 2 years of age and is the most predominant concern in adults [40,41]. Up to 90% of individuals suffering from CF become infected with *P. aeruginosa* during their lifetime, and this organism is the leading cause of morbidity and mortality among those patients. It is the dominant pathogen in chronic lung infection in CF. In the majority of cases, colonization of the CF airway by *P. aeruginosa* leads to a chronic infection that is resistant to antimicrobial therapy [42,43]. Chronic colonization and infection with *P. aeruginosa* is an inevitable reality for the majority of adults with CF, as over 80% of adults over the age of 18 years return positive cultures for *P. aeruginosa*

[44]. The nature of this disease is critical in understanding why *P. aeruginosa* dominates as the primary pathogen in CF patients and so the pathology is addressed below.

The defective gene involved in CF encodes for CFTR resulting in pathological changes in organs that express CFTR, including lungs. In a normal airway epithelial cells, the gene encoding for CFTR regulates the transport of chloride, sodium, and water. Abnormalities of the CFTR gene product lead to a thick and dehydrated mucous secretion that impairs mucociliary clearance of bacterial pathogens [45]. In the normal lung, the mucus layer functions in binding and cleaning inhaled pathogen, and although the bacterial load can be quite high in the upper airways, the lower airways remain free of bacteria [46]. Due to the characteristic thickened mucus associated with CF resulting an inability of ciliary beating to remove the mucus, invading pathogens become trapped in the mucus layer. As a result, a constant presence of bacteria with expression of pathogen-associated molecular patterns (PAMPs) leads to chronic inflammation, consequently damaging the epithelial surface [47-49].

A novel concept of host susceptibility emerged in that the epithelial cells use CFTR as a receptor for internalization of *P. aeruginosa* and subsequent removal of bacteria from the airway surface [50,51]. Accordingly, CFTR is considered as a pattern recognition molecule that extracts *P. aeruginosa* LPS from the organism's surface into epithelial cells [52]. The prevention of CFTR-*P. aeruginosa* interactions leads to decreased bacterial clearance and increased bacterial burden in the lungs.

1.1.2.1 Adaptation During Chronic Infection

CF patients frequently become colonized in the upper airways by environmental isolates of *P. aeruginosa* [53,54]. During the process of infection, a number of

adaptations occur leading to the characteristic persistence and antibiotic resistance of isolates found from chronic infection. Amongst the most common adaptations of *P. aeruginosa* found in CF isolates is the conversion to the mucoid phenotype due to overexpression of alginate [55]. Environmental isolates usually present a non-mucoid phenotype. However, as *P. aeruginosa* penetrates the thickened mucus lining of the airways travelling down the oxygen gradient, an increased expression of alginate and a switch to a mucoid phenotype occur [56,57]. This phenotype often occurs coincidently with the establishment of chronic infection and becomes stabilized by regulatory mutations as described earlier. The mucoid form of *P. aeruginosa* is associated with 90% of *P. aeruginosa* CF infections compared to only 2% of *P. aeruginosa* non-CF infections [58,59]. This phenotype is often coordinately regulated with a loss of flagella by the alternative sigma factor AlgT [60]. The loss of flagella causes not only loss of motility, but also a decreased activation of host inflammatory responses [61].

Other easily identified morphological adaptations of *P. aeruginosa* include the switch from smooth to rough colony morphology and the appearance of small colony variants. The rough colony morphology is representative of strains that have lost the LPS O-antigen [31]. As the O-antigen is the immunodominant portion of the LPS, this adaptation leads to a less virulent phenotype. It also makes rough isolates more susceptible to complement killing and perhaps explains in part why these organisms virtually never cause invasive infections. Modifications to the lipid A moiety of the LPS are also observed. These include the addition of palmitate, aminoarabinose and the retention of 3-hydroxydecanoate [62]. The small colony phenotype is less well understood but is of considerable interest as these isolates exhibit increased antibiotic

resistance. Isolates exhibiting this phenotype have been found to be hyperpiliated with increased abilities in twitching motility and biofilm formation, and with a decreased ability for swimming [63].

Another phenotype of relevance to the resistance to antimicrobial therapy is the hypermutator phenotype, which is frequently observed in CF isolates, but less commonly in nosocomial isolates of *P. aeruginosa* [64]. This phenotype, characterized by up to 1000-fold increased mutation frequency, has been attributed to mutations in genes encoding DNA replication and repair mechanisms, such as *mutS*, *mutL*, and *mutY*. Most importantly, these hypermutator isolates can develop resistance more readily during a course of antimicrobial therapy than do non-mutator isolates. The hypermutator phenotype can give rise to a variety of mixed morphologies within the lung, including those described above [65]. These diverse populations can colonize or infect different compartments within the lung and often have variable antimicrobial susceptibilities with difference virulence properties [66-68].

Comparison of *P. aeruginosa* isolates from the CF lung to strains from non-CF patients clearly shows that CF isolates tend to demonstrate an overproduction of β-lactamase, loss of OprD, and an overproduction of MexXY. This efflux pump overproduction leads to high-level aminoglycoside resistance and the overproduction of this and other efflux systems also lead to quinolone resistance, amongst which MexCD-OprJ is the most frequent [69].

1.1.2.2 Antimicrobial Therapy for Treatment of *P. aeruginosa*

P. aeruginosa isolates from CF patients frequently develop multi-drug resistance. Combination therapy can be used to avoid resistance development and to exploit the

synergistic effects of the bactericidal antibiotics. The use of aerosols allows for drugs to be delivered directly to the lung in CF patients and a number of antibiotics including gentamicin, tobramycin, colistin, ceftazidime, carbenicillin aztreonam, and amikacin have been administered as aerosols to CF patients, although approved formulations and adequate controlled studies have not been performed on most of these [70,71].

1.1.2.2.1 Antimicrobial Therapy for Colonization and Initial Infection

Eradication of *P. aeruginosa* from the CF lung is possible only in the early stages of colonization. At this point, the bacterial load tends to be low, and the organism is non-mucoid and has not begun to undergo significant morphological changes. Aggressive antimicrobial treatment upon first isolation of *P. aeruginosa* has been demonstrated in most cases to delay and occasionally prevent the onset of chronic infections resulting in a better quality of life and a greater life expectancy [72,73]. Successful eradication is judged by the observation of at least three consecutive negative cultures at intervals of at least one month. After one year of negative cultures following the onset of antimicrobial therapy, any isolation of *P. aeruginosa* is considered to represent a new isolate [40]. Aggressive antimicrobial use at the early stage has proven to be successful in certain cases, with a number of patients having remained culture negative for *P. aeruginosa* for several years after treatment [74,75].

1.1.2.2.2 Antimicrobial Therapy for Chronic Infections

Once chronic *P. aeruginosa* infection has been established the high bacterial load present in the lung, as well as the phenotypic changes occurring in the pathogen complicate the antimicrobial therapy. The high bacterial load and thickened mucus are barriers to the attainment of sufficient exposure of the entire bacterial population to

bactericidal concentrations of antibiotics [76]. Administration of insufficient concentrations of antibiotics adds to an increased selective pressure for resistant phenotypes, thereby enhancing the diversity of the population, lending further difficulties to effective treatment [68,77].

Antimicrobial therapy is used during chronic infections in CF for two main purposes: maintenance therapy and treatment of acute exacerbations of infection [78]. Maintenance therapy is recommended for CF patients with chronic *P. aeruginosa* infections in order to reduce bacterial load and maintain overall lung function. Unfortunately, a number of side effects are associated with long-term antimicrobial use including loss of hearing, increased cough, alterations of the voice, and the appearance of antibiotic resistant strains. The use of on/off cycles of intermittent drug administration lead to the reduced occurrence of these side effects [79].

1.1.3. Pathogenesis and Major Virulence Factors

Pathogenesis of *P. aeruginosa* infection is mediated by multiple bacterial virulence factors that facilitate adhesion and/or disrupt host cell signaling pathways while targeting the extracellular matrix (Figure 1). *P. aeruginosa* stands out as a unique and threating organism as it is capable of causing severe invasive disease and of evading immune defenses causing persisting infections that are nearly impossible to eradicate [29]. The subsequent tissue damage, invasion, and dissemination of *P. aeruginosa* are likely attributed to the many virulence factors it produces. These virulence factors play an initial role in motility and adhesion to the epithelium. These virulence factors are thought to be critical for maximum virulence of *P. aeruginosa*; however, based on observations of diverse plant and animal models, the relative contribution of any given factor may vary

with the type of infection [80-84]. Several of these virulence factors have also been studied for their roles as potential vaccine candidates although there is currently no any generally accepted vaccine. The following section briefly outlines several prominent virulence factors produced by *P. aeruginosa* and their proposed roles in contributing to disease.

1.1.3.1 Lipopolysaccharide

The LPS is a predominant component of the outer membrane of *P. aeruginosa*. Bacterial LPS typically consists of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating core oligosaccharide, and a distal polysaccharide (or O-antigen) [85]. The composition of O-antigen determines the serotype of *P. aeruginosa* isolates and there are currently 20 serotypes based on serological reactivity of the O-antigen [86]. LPS plays a prominent role in the activation of the host innate and adaptive (or acquired) immune responses; LPS also causes dysregulated inflammatory responses that contribute to morbidity and mortality [87].

Recognition of LPS occurs largely through the TLR4–MD2–CD14 complex, which is present on many cell types including macrophages and dendritic cells. Recognition of lipid A also requires an accessory protein, LPS-binding protein (LBP), which converts oligomeric micelles of LPS to a monomer for delivery to CD14, which is a high-affinity membrane protein that can also circulate in a soluble form [88-92]. In addition, NLRs regulate both inflammation and pyroptosis. The activation of NLRs results in an assembly of complex structures called inflammasomes [93]. The NLRP1 inflammasome was first described in 2002 in human monocytes as a molecular compound that responds to LPS [94]. Many stimuli that trigger the assembly of the inflammasomes have been described.

LPS's are also reported to activate NLRP3 when administered in the presence of ATP [95], as well as NLRP2 [96]. A number of LPS vaccines have been investigated for use in CF patients in phase II and III clinical trials; however, these have not been successful [97-101]. The LPS based vaccines provided little immunity and did not appear to protect the patients from infection with *P. aeruginosa* [102].

1.1.3.2 Flagellum

The single unsheathed polar flagellum of *P. aeruginosa* is responsible for the swimming motility of this organism [103]. Nonetheless, its role in virulence goes beyond simple motility. Flagellar proteins have been shown to play critical roles in attachment, invasion, biofilm formation, and mediating inflammatory responses. Flagellar protein synthesis, assembly and regulation involves more than 40 genes and is intricately controlled through transcriptional and post-translational events by the four primary regulators RpoN, FleQ, FleR and FliA [104].

Non-flagellated mutants are often isolated from chronic infections in CF patients [105] due to the repressor activity of AlgT, which acts on the FleQ regulator [60]. The loss of flagella in these isolates is believed to be useful for the invasion of the host immune system. Flagellin mediates the inflammatory response via the activation of the innate immune system, through its specific interaction with a number of pattern recognition receptors (PRRs) of the host [106]. Flagellin is recognized by both TLR5 [107] and NLRC4 [108,109]; as well NLRP3 [110]. However, different amino acid residues of flagellin are critical for sensing by NLRC4 or TLR5 [108]. Moreover, cytosolic delivery of *P. aeruginosa* flagellin is required for the activation of NLRC4 [111]. Surprisingly, the NLRC4 inflammasome can be activated independently of

flagellin. The flagellin-deficient strains of *P. aeruginosa* can efficiently activate caspase-1 in an NLRC4-dependent manner. This discrepancy in the requirement for flagellin in NLRC4 inflammasome activation was recently explained in an elegant study by Maio and colleagues [112]. They found that the NLRC4 inflammasome was activated in response to the basal body rod component of the T3SS apparatus from *P. aeruginosa* (Pscl), as well as from other microorganisms, such as *S. typhimurium* (PrgJ), *Burkholderia pseudomallei* (BsaK), *Escherichia coli* (EprJ and EscI), *S. flexneri* (MxiL) [112]. These rod proteins contain a sequence motif that resembles the one found in flagellin; hence, NLRC4 is activated by either of these similar stimuli. Furthermore, flagellar vaccines have been investigated in pre-clinical studies in mouse models and have reached phase III clinical trials for CF patients; however, limited protection was observed with a monovalent vaccine and development of a bivalent vaccine has been terminated [97].

1.1.3.3 Type IV Pili

The type IV pili of *P. aeruginosa* have a role in adhesion to many cell types and this is likely important in such phenomena as tissue tropism (attachment to particular tissues), initiation of biofilm formation and non-opsonic phagocytosis, which is mediated by phagocyte receptors that recognize corresponding adhesins on microbial surfaces [113-115]. Several studies have found a direct correlation between the presence of glycosphingolipids on host cells and *P. aeruginosa* adherence, thus demonstrating thir role as bacterial receptors. In particular, *P. aeruginosa* pili bind to the glycosphingolipid contained within host epithelial cell membranes, ganglio-N-tetraosylceramide (asialo-GM1) [116]. The interaction of pili and asialo-GM1 is followed by the internalization of *P. aeruginosa* in

host epithelial cells. In addition, these pili also mediate twitching motility found to be important in the formation of biofilms *in vitro* [117]; as well as in the initiation of bacterial dissemination from an initial point of colonization [118,119]. Although more than 50 genes have been identified to play either a direct or indirect role in the synthesis, function and control of the type IV pili of *P. aeruginosa*, the pili are composed of a single type IV pilin protein encoded by *pilA* [120]. Five alleles of *pilA* have been identified with group I pili being the most prevalent in both lung CF and environmental isolates [121]. *P. aeruginosa* pilin, the major component of the type IV bacterial pili, is identified as an inflammasome-activating factor; as purified pilin activated caspase-1 and lead to secretion of mature IL-1β [122].

1.1.3.4 Type III Secretion System

P. aeruginosa has a variety of secretion systems of which at least four likely play a role in virulence (Type I, II, III, and IV). One of the most intriguing is T3SS that involves a flagellum-basal-body related system for delivering proteins directly from the cytoplasm of *P. aeruginosa* into the cytosol of host cells. A functional T3SS contributes to the successful phagocytosis evasion by *P. aeruginosa* as well as to the damage to host tissues, promotion of immune avoidance and bacterial dissemination. The T3SS of *P. aeruginosa* delivers up to four cytotoxins, ExoS, ExoT, ExoU and ExoY, directly to host cells [123-125].

ExoS and ExoT are bifunctional cytotoxins that possess both Rho GTPase-activating protein and ADP ribosyltransferase activities. These molecules can inhibit phagocytosis by disrupting actin cytoskeletal rearrangement, focal adhesions and signal transduction [126]. Moreover, ExoU is a phospholipase, which contributes directly to

acute cytotoxicity towards epithelial cells and macrophages; while ExoY is an adenylate cyclase that affects intracellular cAMP levels and cytoskeleton reorganization [127,128]. Recent evidence has implicated a role of T3SS in *P. aeruginosa* pathogenesis in humans. The presence of large amount of T3SS products, particularly ExoU, in *P. aeruginosa* cultures isolated from intubated patients was linked to increased mortality regardless of whether these patients had symptoms or confirmation of VAP [129]. Also, *P. aeruginosa* T3SS activates the NLR inflammasome. However, in the absence of any of the known effector proteins, *P. aeruginosa* T3SS apparatus is sufficient to trigger the activation of caspase-1 by the inflammasome via NLRC4 [130]. In another words, a functional T3SS is critical for the induction of caspase-1 activity, IL-1β secretion and cell death, whereas the effectors ExoS, ExoT and ExoY are dispensable [108].

1.1.3.5 Exotoxin A

There are several critical virulence factors that are secreted through Type II secretion mechanism, which use a pilus-like apparatus to secrete proteins into the extracellular environment, including exotoxin A, lipase, phospholipase, alkaline phosphatase, and protease; animal experiments have indicated the significant role of these factors in a model of infection [131]. For example, exotoxin A has been demonstrated to be involved in local tissue damage and invasion. This cytotoxin is encoded by the gene toxA and has been found to be present in most clinical isolates of *P. aeruginosa*, although its role in virulence is poorly understood [131]. Besides, exotoxin A enters host cells by receptor-mediated endocytosis and catalyzes the ADP-ribosylation of eukaryotic elongation factor-2 (EF-2) [132]. The EF-2 inhibits protein synthesis, ultimately leading to cellular death.

1.1.3.6 Proteases

P. aeruginosa produces several secreted proteases including the zinc metalloprotease (elastase) LasB, the metalloendopeptidase LasA, and alkaline protease. These proteases work in a concerted fashion to destroy host tissue and hence they play a significant role in both acute lung infections and in burn wound infections [133-135]. A definite role of these destructive proteases in acute infections has been established. LasA and LasB elastases have also been found in the sputum of CF patients suffering from exacerbations of pulmonary infection [136,137]; yet, their role in chronic infection is not well understood.

1.1.3.7 Alginate

P. aeruginosa can produce a mucoid exopolysaccharide capsule, comprised of alginate, an acetylated random co-polymer of β 1-4 linked D-mannuronic acid (poly-M) and L-guluronic acid [138]. The overproduction of alginate is believed to play a role in cell adherence within the CF lung and is also thought to be involved in resistance to host defense by reducing susceptibility to phagocytosis [139], also in resistance to antibiotics. The small minority of CF patients, who are carrying only nonmucoid *P. aeruginosa*, have significantly better lung function over time compared to those patients infected with mucoid *P. aeruginosa* [140]. The tendency of *P. aeruginosa* to change to a mucoid phenotype is one of the most striking and clinically relevant features of infection by this bacterium. Additionally, poly-M shares with LPS the ability to stimulate human monocytes for CD14 cytokines production; in a CD14-dependent manner [141]. Involvement of TLR2 and TLR4 in cell activation by poly-M has been found in primary murine macrophages [142].

1.1.3.8 Quorum Sensing

Quorum sensing is a mechanism of bacterial “cell-to-cell” communication via diffusible chemical compounds. A critical number of bacteria (the quorum) are required to produce a sufficient amount of a secreted signal molecule (termed an autoinducer) to trigger expression of a large regulon [143-145]. Quorum sensing and biofilm development are two social phenomena exhibited by bacteria. The connection between quorum sensing and biofilms has been named sociomicrobiology [146,147]. In addition, *P. aeruginosa* is regarded as a "model organism" in the quorum sensing field, which has been studied in most detail. Quorum sensing is known to control a number of bacterial genes. More than 300 genes are regulated via quorum sensing in *P. aeruginosa* [148].

The most common class of autoinducers used by Gram-negative bacteria is acyl-homoserine lactones (AHL), which diffuse freely across bacterial membranes. AHL signals produced by *P. aeruginosa* are oxohexanoyl-homoserine lactone and butanoyl-homoserine lactone [149,150]. AHL signals are produced by AHL synthase (LasI/RhlI), which diffuse into the environment. Increasing in bacterial density during infection leads to an increase in autoinducer concentration. When autoinducer reaches a particular threshold, it subsequently binds to transcriptional activator (LasR/RhlR) forming a complex that activates genes involved in biofilm formation and coding virulence factors [151-153]. The production of virulence factors, such as extracellular enzymes and cellular lysins (e.g., rhamnolipid) are important for the pathogenesis of infections as a protective shield against phagocytes [154-156]. Quorum sensing has been shown to determine the tolerance of *P. aeruginosa* biofilms to antibiotic therapy [157].

Recent advances in the understanding of quorum sensing in *P. aeruginosa* have generated interest in using quorum sensing as a target for therapeutics. The macrolide antibiotic, azithromycin, has been a promising candidate in this regard as it has been demonstrated to be capable of both penetrating biofilms and interfering with quorum sensing [158].

1.1.3.9 Biofilm Formation

P. aeruginosa is capable of forming complex structures called biofilms. Resistance to antimicrobial agents is the most important feature of biofilm infections. Biofilm development is a complex process partly controlled by quorum sensing signals (Figure 2). Furthermore, a variety of components play a role in the initial attachment of cells to the surface and development of biofilm matrix including extracellular DNA (eDNA) [159], exopolysaccharide (Psl, Pel, and alginate) [160,161], iron siderophore pyoverdine, biosurfactant rhamnolipid [162], and proteinaceous surface appendages such as type IV pili, flagella [119], Cup fimbria [163]. There are still numerous factors that are involved in biofilm formation process and dispersion, which are related to signals, regulatory networks, and materials, reviewed elsewhere [161,162,164].

During biofilm formation, cell differentiation occurs, and oxygen and water-filled channels are formed to provide nutrition to the deep-rooted cells of the mature biofilm [165-168]. *P. aeruginosa* has been demonstrated to form biofilms on a variety of indwelling medical devices [169,170]. It is particularly problematic for patients requiring mechanical ventilation and catheterization, as the surfaces of medical devices can readily develop *P. aeruginosa* biofilms that are difficult to remove. Also, *P. aeruginosa* has been demonstrated to grow as a biofilm within the body particularly at the site of burn wounds. It

has been proposed that *P. aeruginosa* exists as a biofilm in the CF lung [9,171] and this has been observed in a mouse model of CF lung infection [172].

In addition to evasion of the host immune system, a highly resistant nature of biofilms to killing by bactericidal antibiotics contributes to bacterial persistence in chronic infections [173]. It has been demonstrated that cells growing in a biofilm can be up to 1000 fold more resistant to antibiotics than free-swimming, planktonic cells [174]. Biofilms present not only a diffusion barrier to antibiotics, but also the cells in a biofilm have been demonstrated to have significantly different gene expression compared to their planktonic counterparts [175].

1.1.3.10 Type VI Secretion Systems

Bacterial pathogens frequently possess a number of secretion systems that function to translocate protein secretion. The T6SS represents one of the most recently recognized examples of these secretion systems. An interest in T6SS has led to its rapid study in *P. aeruginosa* in term of structure, mechanical function, assembly, and regulation of secretion [176,177]. *P. aeruginosa* T6SS provides defense against other bacteria in the environment [177,178] and facilitates interactions with other eukaryotic [179]. *P. aeruginosa* encodes three distinct T6SS, which are known as H1-, H2-, and H3-T6SS, each involved in bacterium's interaction with other organisms. The H1-T6SS delivers at least six toxic effectors into host bacteria and is a model for studying physiological function of T6SS antimicrobial activity [177,178,180]. H2- and H3-T6SS have a dual role allowing interaction with both eukaryotic and prokaryotic target cells. The antibacterial activities are mediated through H2-T6SS-dependent phospholipase D (PLD) PldA and H3-T6SS-dependent PldB. Both T6SS effectors, PldA and PldB can degrade membrane

phospholipids, resulting in antibacterial activity. T6SSs, H2-T6SS-dependent PldA and H3-T6SS-dependent PldB, have also been linked to *P. aeruginosa* immune evasion by promoting internalization into human epithelial cells [179]. Interestingly, mutations in the catalytic domains of both PldA and PldB reduced *P. aeruginosa* internalization into epithelial cells, which shows that phospholipase activity is essential for invasion of the mammalian epithelium by *P. aeruginosa* [179].

Previous works have shown that the internalization of *P. aeruginosa* is dependent on the activation of the eukaryotic phosphoinositide 3-kinase (PI3K) that results in AKT phosphorylation in presence of phosphatidic acid, subsequent actin rearrangement and protrusion formation [179,181,182]. In addition, PI3K/AKT signaling pathway is crucial for some processes including cell growth, proliferation, and programmed cell death [183]. Notably, epithelial cells that were infected with *P. aeruginosa* mutants (PldA, PldB, H2-T6SS, or H3-T6SS deficient) displayed reduced levels of AKT phosphorylation compared to the wild type strain. Furthermore, PldA and PldB were shown to bind to AKT, and both PldA-AKT and PldB-AKT complexes localized close to the epithelial cell plasma membrane [179]. These data suggest that PldA and PldB have a central role in the activation of the PI3K-AKT signaling pathway to promote the invasion of epithelial cells by *P. aeruginosa*. Whereas *P. aeruginosa* is known to colonize the lungs of CF patients, previous studies have indicated that PldB and H3-T6SS loci are both up-regulated under low-oxygen conditions [184] and also during biofilm formation [185]. For this reason, induction of both PldB and H3-T6SS in these conditions may allow better colonization of the lung epithelium through invasion. These findings reveal a function for the *P. aeruginosa* H3-T6SS effector PldB and show that PldB and PldA can influence both

bacterial competition and interaction with mammalian hosts. T6SSs are promising targets for the development of new approaches to diagnosis, vaccine development and antimicrobial drug design [186,187].

1.1.3.11. Oxidant Generation in the Airspace

Oxidative stress refers to as an imbalance in the redox status of the cell favoring an oxidizing environment. Extensive reactive oxygen species (ROS) production leads to the depletion of antioxidants and results in cellular damage. In particular, ROS can damage DNA strands by reacting with base pairs and the deoxyribose phosphate backbone of DNA, the primary target of radical damage [188]. Without the protection by antioxidants, ROS can also initiate lipid peroxidation of polyunsaturated fatty acid components of cell membrane phospholipids, affecting cellular integrity [189]. Amino acids can also be damaged by ROS, leading to protein denaturation and enzyme deactivation [188,189]. When left unmanaged, oxidative stress can eventually lead to cell death.

During the infectious process, *P. aeruginosa* induces ROS production within epithelial cells in a few ways. Following its secretion into the microenvironment, pyocyanin permeates the epithelial cell membrane and directly oxidizes intracellular pools of NADPH and glutathione, producing superoxide and downstream ROS [190]. Recognition of *P. aeruginosa* LPS by the epithelial cells leads to ROS production through protein kinase C (PKC)-NADPH oxidase signaling pathway in human epithelial cells [191]. Other potential sources of ROS are derived from the activated epithelium via induction of the mitochondrial electron transport chain, cytochrome p450, and xanthine oxidase. In the case of mechanical ventilation, the introduction of excess oxygen can also fuel the production of ROS [192]. In acute lung injury, however, stimulated phagocytes

produce the majority of ROS [193]. Overwhelming oxidant injury may lead to alveolar collapse and extensive fibrotic scarring, impairing gas exchange between the affected airways and the capillary system [193].

1.1.4. Antimicrobial Resistance

P. aeruginosa can be an especially challenging organism to treat once infection has been established as it is intrinsically resistant to many of the available antibiotics. Four mechanisms have been studied by which *P. aeruginosa* resist the action of antibiotics. The outer membrane of *P. aeruginosa* restrictes the penetration of antibiotics. The efficient removal of antibiotics molecules by efflux pumps before acting on their targets occurs. *P. aeruginosa* has the genetic capacity to inactivate and modify antibiotics. This bacterium can become resistant through mutational changes in antibiotic's targets [6]. Consequently, *P. aeruginosa* has now achieved the status of the superbug. This section will provide an overview of the main mechanisms of resistance present in clinical isolates of *P. aeruginosa*.

1.1.4.1 Intrinsic Resistance

P. aeruginosa exhibits intrinsic resistance to almost all of the available antibiotics, indicating that the one strain possesses number of genetic mechanisms that contribute to reducing susceptibility of the organisms. One of the major factors contributing to this intrinsic resistance is the low permeability of the outer membrane. The outer membrane is essential for passively determining the rate of uptake of antibiotics and small molecules [10]. However, by itself this is insufficient to mediate significant resistance, and antibiotics will equilibrate across the outer membrane. Thus, intrinsic resistance arises from the combination of slow uptake and secondary mechanisms that benefit from this

slow uptake including degradative enzymes such as periplasmic β-lactamase and particularly multidrug efflux systems. There are at least four antibiotic efflux systems that have been described including MexAB-OprM and MexXY-OprM, MexCD-OprI, and MexEF-oprN elsewhere [6,10,194,195].

The *P. aeruginosa* outer membrane is an asymmetric membrane composed of an inner leaflet of phospholipids, predominantly phosphatidylethanolamine, and outer layer of polyanionic LPS. The latter presents a negatively charged surface, which, together with the divalent cations bridging the individual LPS molecules, forms a matrix around the cell that is relatively impermeable to polar compounds except polycations [196]. Multidrug efflux pumps also mediated resistance to many classes of antibiotics. The *P. aeruginosa* genome contains a large number of drug efflux systems [5], which have been categorized into five superfamilies including: the small multidrug resistance family, the ATP-binding cassette family, the multidrug and toxic compound extrusion family, the resistance-nodulation-cell division family, and major facilitator superfamily [195,197-200].

P. aeruginosa also expresses periplasmic β-lactamases to degrade β-lactan antibiotics. The β-lactamases are hydrolyzing enzymes that cleave the lactam ring of penicillins, carbapenems, cephalosporins and monobactams, thus leading to inactivation of the antibiotic [201-203]. In *P. aeruginosa*, this activity is due to a chromosomally encoded AmpC β-lactamase. The AmpC β-lactamase of *P. aeruginosa* can degrade and contribute to intrinsic resistance to ticarcillin, piperacillin and the third-generation cephalosporins. It is strongly induced by carbapenems, particularly imipenem, although these inducing carbapenems are stable against its hydrolytic activity [204].

1.1.4.2 Adaptive Resistance

Discrepancies between in *in vitro* susceptibility of *P. aeruginosa* isolates and treatment outcomes in CF patients have been observed, and can be attributed to the phenomenon of adaptive resistance. Adaptive resistance occurs when cell populations are exposed to non-lethal concentrations of antibiotic and then undergo specific changes in gene expression that result in reduced susceptibility. It is a form of inducible resistance that does not require the presence of mutations; it has been demonstrated *in vitro* using CF isolates and in mouse models, when isolates were pre-incubated with subinhibitory concentrations of antibiotics [205-207]. However, the concern arises that this induction may allow small population to survive and acquire stably resistant mutations. Similarly, polymyxin susceptibility in *P. aeruginosa* is associated with the LPS structure, which is determined by arnBCADTEF and modulated by PhoPQ and PmrAB [208]. Adaptive resistance to polymyxins and antimicrobial peptides has been shown to occur through altered expression of the PhoPQ and PmrAB systems in response to these agents. This also leads to modulation of Lipid A fatty acid composition [209], which ultimately affects resistance to not only polymyxin and antimicrobial peptides, but also the aminoglycosides, which rely on the LPS binding for self-promoted uptake.

1.1.4.3 *P. aeruginosa* as a Superbug

The accumulation of multiple resistance mechanisms in clinical isolates of *P. aeruginosa* resulted in strains that are resistat to all available antibiotics. This pandrug resistance, which means resistant to all antimicrobial agents, together with high attributable mortality, has thrust *P. aeruginosa* into the spotlight as an emerging superbug. According to report by the National Nosocomial Infections Surveillance (NNIS) System,

which focused on nosocomial infections in ICU, not only were resistance rates increasing, but the incidence of occurrence of most infection types was also increasing [210]. In 2003, the NNIS reported a 9% increase in resistance to the third generation cephalosporins, a 15% increase in ciprofloxacin resistance, and most alarming, a 47% increase in imipenem resistance over a five year period. According to the European Antimicrobial Resistance Surveillance System, 18% of *P. aeruginosa* isolates were multidrug resistant, with 6% of all isolates being resistant to piperacillin, ceftazidime, fluoroquinolones, aminoglycosides and carbapenems [211]. The drug of last resort for infections with multidrug resistant *P. aeruginosa* is colistin (polymyxin E), and while resistance rates remain low (approximately 1% in most countries), mortality of 80% has been observed for infections caused by colistin resistant Gram-negative bacilli [212].

1.1.5. Conclusion

In conclusion, it would be impossible to remove *P. aeruginosa* from the environment, even from the internal environment of the hospitals because it is so hardy and metabolically versatile. However, the last two decades have seen a remarkable addition in active medication and therapy to the treatment of CF lung infection. These therapies have enhanced the overall health of patients with CF and this is apparently a partial reason that demanded survival has increased. However, these therapies do not offer a cure and they primarily target downstream complications of the pathophysiology of CF lung infection, meaning that patients continue to have morbidity. However, innate immunity is critical in protecting the host from bacterial invasion, but at the same time it can directly & indirectly damage tissues. In addition, these therapies add to a considerable treatment burden and are thus also associated with poor adherence. What is

more, it appears likely that the antibiotic resistance will continue to be a problem in dealing with *P. aeruginosa* infections. The fundamental issues underlying this problem are the conditions of the patients that are prone to such infections, and, the high intrinsic resistance of this bacterium, which has remained constant. Chronic *P. aeruginosa* airway infection and the accompanying inflammatory response are clearly the significant clinical problems for CF patients today. No doubt, there is an urgent need for alternative therapeutic strategy with *P. aeruginosa* infection.

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Figure Legend

Figure 1 *P. aeruginosa* Pathogenesis and Major Virulence Factors

Pathogenesis in *P. aeruginosa* infection is mediated by various adhesins and secreted toxins, proteases, effector proteins and pigments that facilitate adhesion, modulate or disrupt host cell signaling pathways, and target the extracellular matrix. Figure has been recreated from Hauser and Ozer [213]. Abbreviations: **ADP**, adenosine diphosphate; **Asialo-GM1**, asialo-gangliotetraocyl ceramide 1; **EF2**, elongation factor 2; **FpvA**, feeric pyoverdine receptor; **PA,** phosphatidic acid; **RAS**, ribosyltransferase; **SOD1**, superoxide dismutase 1; **14-3-3**, 14-3-3 protein family.

Figure 2 Development of a *P. aeruginosa* biofilm.

Biofilm formation starts with the attachment of free-swimming bacteria (planktonic) to a surface via their type IV pili and flagellum, followed by twitching motility and the formation of microcolonies; then quorum sensing signals begin to accumulate. Once a critical threshold of quorum sensing signals is reached, microcolonies increased in the extracellular matrix. Cells enter a sessile phase of growth and become highly resistant to antimicrobials and then evolve into mature biofilms. Biofilm architecture depends on the production of the biofilm matrix, which consists of the polysaccharides Pel (synthesized by PelA–PelG), Psl (arranged in a helical pattern around cells) and alginate, extracellular DNA (eDNA), and proteins, including the CupA, CupB and CupC fimbriae, which mediate bacterial attachment during initial biofilm formation, and the lectin LecB. The extracellular polymeric matrix delays diffusion of some antibiotics into the biofilm. A gradient of oxygen and nutrients results in the formation of distinct bacterial

subpopulations that vary in their susceptibility to antibiotics; exposure to β-lactams or colistin can cause the production of resistance factors (AmpC β-lactamase and MexA–MexB–OprM efflux pumps). Rhamnolipids at the bacterial surface cause necrosis of neutrophils. Finally, planktonic bacteria are released from parts of a mature biofilm. Individual cells and small microcolonies slough from the mature biofilm initiating further biofilm development. Figure 2 bacteria has been recreated from Hauser and Ozer [213].

Figure 1

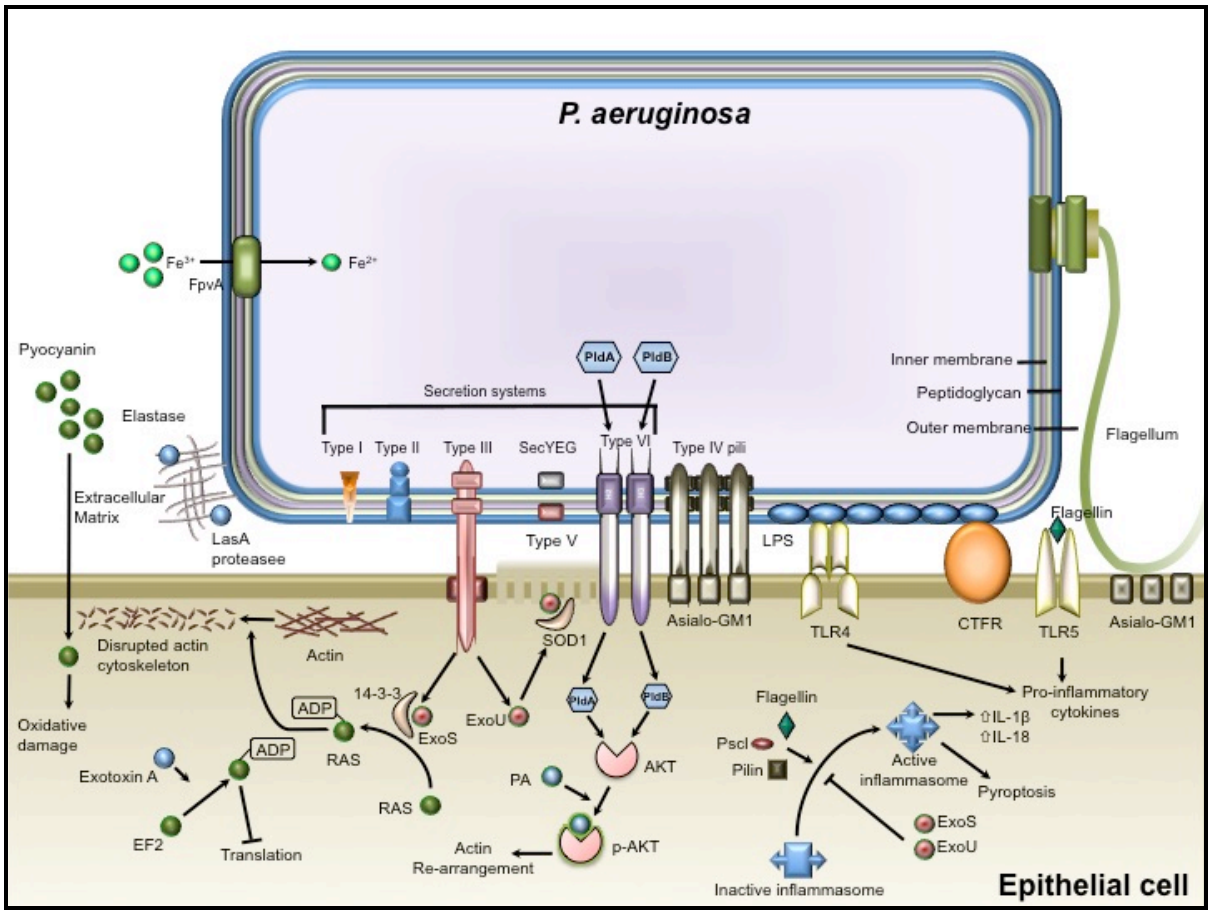
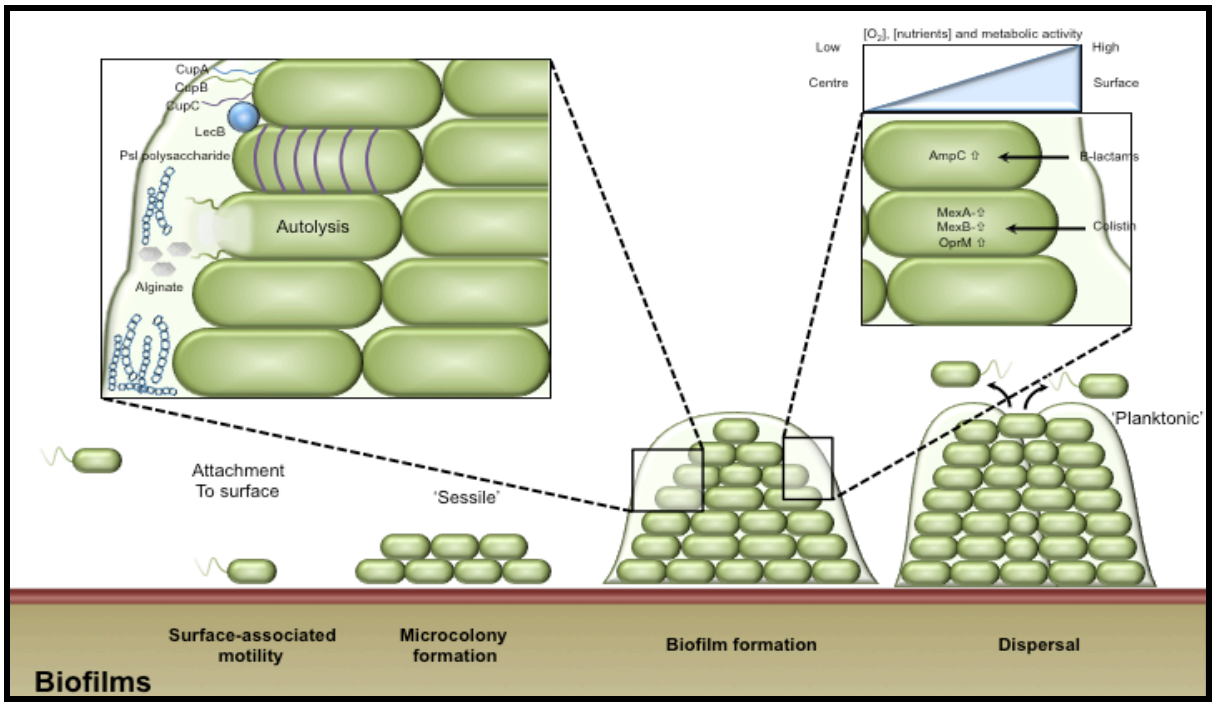

P. aeruginosa
Fe3+
FpvA
Fe2+
Pyocyanin
Elastase
Extracellular Matrix
LasA protease
PldA
PldB
Secretion systems
Type I
Type II
Type III
SecYEG
Type V
Type VI
Type IV pili
Inner membrane
Peptidoglycan
Outer membrane
Flagellum
LPS
Flagellin
Asialo-GM1
TLR4
CTFR
TLR5
Asialo-GM1
Disrupted actin cytoskeleton
Actin
SOD1
Oxidative damage
ADP
14-3-3
ExoS
ExoU
RAS
Exotoxin A
EF2
Translation
PA
AKT
p-AKT
Actin Re-arrangement
Pro-inflammatory cytokines
⇧IL-1β
⇧IL-18
Pyroptosis
Pscl
Pilin
Active inflammasome
Inactive inflammasome
Epithelial cell

Figure 2


CupA
CupB
CupC
LecB
Psl polysaccharide
Autolysis
Alginate
[O2], [nutrients] and metabolic activity
Low
High
Centre
Surface
AmpC ⇧
β-lactams
MexA-⇧
MexB-⇧
OprM ⇧
Colistin
'Planktonic'
Attachment
To surface
'Sessile'
Surface-associated motility
Microcolony formation
Biofilm formation
Dispersal
Biofilms

1.2 NOD-like receptor(s) and host immune responses with *Pseudomonas aeruginosa* infection

Published Review: Journal of Inflammation Research

Author: Alaa Alhazmi

Abstract

Introduction: Molecular mechanisms underlying the interactions between *Pseudomonas aeruginosa*, the common opportunistic pathogen in cystic fibrosis individuals, and host induce a number of marked inflammatory responses and associate with complex therapeutic problems due to bacterial resistance to antibiotics in chronic stage of infection. **Methods:** *P. aeruginosa* is recognized by number of pattern recognition receptors (PRRs); NOD-like receptors (NLRs) are a class of PRRs, which can recognize a variety of endogenous and exogenous ligands, thereby playing a critical role in innate immunity. **Results:** NLR activation initiates forming of a multi-protein complex called inflammasome that induces activation of caspase-1 and results in cleavage of pro-inflammatory cytokines interleukin (IL)-1β and IL-18. When the IL-1β is secreted excessively, this causes tissue damage and extensive inflammatory responses that are potentially hazardous for the host. **Conclusions:** Recent evidence has laid out inflammasome-forming NLR far beyond inflammation. This review summarizes current knowledge regarding the various roles played by different NLRs and associated down-signals, either in recognition of *P. aeruginosa* or may be associated with such bacterial

pathogen infection, which may relate to for the complexity of lung diseases caused by *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, Infection, Inflammation, NOD-like receptors, Caspase-1

1.2.1 Introduction

Host innate immunity, with its intense concern over how microbes are sensed, is very much linked to microbial pathogenesis. Innate immunity, as a first-line host defense, recognizes either pathogen- or danger-associated molecular pattern molecules (PAMPs and DAMPs, respectively), by the engagement of pattern recognition receptors (PRRs) [1,2]. Thus far, a large group of PRRs have been shown to mediate immune responses. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are expressed on both cell surface and endosomes [3,4]. RIG-like receptors (RLRs) and AIM2-like receptors (ALRs) are both expressed in the cytoplasm [5]. NOD-like receptors (NLRs) are expressed in the nucleus and cytoplasm [6,7]. As well as some other recently identified PRRs for intracellular pathogens [8].

While the role of TLRs in bacterial immunity has been relatively well studied, the contribution of NLRs is less defined. Activation of NLRs have recently been recognized as an essential part of the innate immunity and, consequently, mediate host cellular responses leading to inflammation [1]. NLRs are expressed in many different species such as rat, mouse, cattle, and chimpanzee [9-11], but have been studied more extensively in humans. Structurally, NLRs are composed of a conserved central domain that mediates nucleotide binding and oligomerization, a C-terminal leucine-rich domain (LRR) that

senses NLR agonists, and a N-terminal region required for protein-protein interaction. Human NLR family is composed of 22 known members to date (Table 1) and classified into number of subfamilies based on domain type [1,12]. The activation of NLRs, results in a protein conformation change. This allows the NLR to interact with target proteins (ASC speck and pro-caspase-1) to assemble complex structures called inflammasome [13], which have the ability to either positively or negatively regulate inflammatory responses (Figure 1). Inflammasome is a high-molecular weight signaling platform required for the activation of caspase-1 (also known as interleukin-1 converting enzyme or ICE), which is expressed in various cells in an inactivated form (46 kDa) for maturation of pro-interleukin (IL)-1β and pro-IL-18 into biologically active molecules to evoke inflammatory response [2,14,15]. A general scheme for inflammasome-forming NLR activation is presented in Figure 2. As NLRs have only been studied during the last decade, specific molecular mechanisms of their activation remain largely undefined [1,16]. Inconsistencies in the naming of several NLR members have led to confusion and the work by Ting *et. al.* has proposed a method of nomenclature based on structure [17]. All naming of NLRs in this review follow that recommendation.

While inflammasome-forming NLRs activation initiates a robust innate immune response [1], its activation regulates homeostatic processes the same as inflammation during infection and tissue injury, which is defined as regulatory NLRs. Over the last decade, significant progress has been made in identifying general characteristics of NLR family members. However, despite this progress, many of the identified NLRs lack significant mechanistic and functional insight [18]. Several questions are outstanding regarding the clinical relevance and therapeutic potential of NLRs in human disease,

mostly in the specificity of the mechanisms associated with bacterial pathogen recognition [18]. While the role of the NLRs in response to an opportunistic pathogen, such *P. aeruginosa*, is still an emerging area of study, especially for some regulatory NLRs, this review focuses on the current understanding related to inflammatory-mediated NLRs, unique regulatory NLRs, and emerging concepts associated with their function in bacterial innate immunity.

1.2.2 *P. aeruginosa* as an opportunistic pathogen in Cystic Fibrosis patients

P. aeruginosa is an opportunistic pathogen and a leading cause of morbidity and mortality in cystic fibrosis (CF), which can colonize the lung of CF individuals [19,20]. This bacterial pathogen is equipped with multiple virulence factors that allow it to adhere, infect, invade, adapt, and develop resistance against therapeutic agents [21]. *P. aeruginosa* is also highly adaptable in the CF host lungs with a hypermutable genome [22]. Over the course of *in vivo* infection, *P. aeruginosa* accumulates multiple loss-of-function mutations and mutated bacteria have been isolated from chronically infected CF patient lungs [22-24]. *P. aeruginosa* becomes challenging to treat, as a result of its antibiotic resistance [25,26]. *P. aeruginosa* has proven to be an adaptable pathogen that ensures it persists in CF patients; hence, this persistent bacterial infection underlies the chronic lung infection and continues to provoke the immune responses that CF patients experience [27]. However, bacterial inflammation, the process aimed at restoring homeostasis after an infection, can be more damaging than the infection itself if uncontrolled, excessive, or prolonged and may cause tissue damage, which eventually can interfere with bacterial clearance. Therefore, understanding the molecular mechanisms underlying the host-pathogen interaction and the persistence of *P.*

aeruginosa infection, is a primary step for changing the natural course of this disease. NLRs could be the axis point to attenuate inflammatory responses in *P. aeruginosa*-infected cells and thus, a number of studies have been conducted from a variety of perspectives including: 1) testing *P. aeruginosa* for potency to activate NLRs in leukocytes or respiratory epithelial cells, 2) bacterial challenges in mice deficient in particular NLR gene(s) or signaling molecules, and 3) bacterial challenges of cells or mice with deficiencies in cystic fibrosis transmembrane conductance regulator (CFTR) gene, sometimes combined with deficiencies in NLRs. In this review, NLRs will be considered as a therapeutic target capable of protecting the lung damage during infection.

1.2.3 NLR Signaling

1.2.3.1 Activation of Caspase-1

Caspase-1 was initially identified as the protease responsible for cleave of pro-IL-1β and induce apoptosis. Deeper understanding of this process was provided by the discovery that NLRP3 and ASC are required for the activation of caspase-1 in response to ATP and certain bacterial pore-forming toxins [28]. The mechanism by which caspase-1 is activated in response to infection or tissue damage was found to be regulated by an inflammasome, which consists of a NLR family member, ASC, and an inactive caspase-1 precursor (pro-caspase-1), followed by production of biologically active IL-1β and IL-18 [16,29-32].

There is a discrepancy related to caspase-1 concentration; it has been demonstrated that caspase-1 has low substrate specificity at high concentrations, which is drastically increased by lowering its concentration. Also, it has been reported that the half-life of active caspase-1 is very low comparing to other caspases [33]. Interestingly, pro-IL-1β

and pro-IL-18 are not the only substrates of active caspase-1 since several proteomics approaches led to the identification of novel caspase-1 cleavage products [33-36] (Figure 3). Active caspase-1 cleaves several enzymes required for glycolysis, which is essential for the synthesis of ATP as required for macrophage survival and activation, such as aldolase, GAPDH, triose-phosphate isomerase, and α-enolase [36]. However, whether involvement of caspase-1 with glycolysis contributes to host immune response or plays an important role in pathogenesis, is not known. Interestingly, executioner caspase-7 is involved in apoptosis pathway and it has been shown that caspase-1 activate caspase-7 in both *in vitro* and *in vivo* [34]. This cleavage requires expression of NLRP3 and NLRC4 when macrophages are stimulated with NLRP3 or NLRC4 activators, respectively [34]. This induction of expression of NF-κB target genes by inflammasome-induced caspase-7 activation points into a different direction, which results in translocation of caspase-7 to the nucleus, where it cleaves PARP1. This induces PARP1 release from chromatin and eventually induces the expression of NF-κB gene [37].

Although capsase-1 supports cell death of immune cells infected by *Salmonella* [38], it can also support survival and confer resistance to pathogenic bacteria [39,40]. Some bacteria express pore-forming toxins as virulence factors, which create holes of various sizes in the plasma membrane of the host cell. These pores lead to the efflux of intracellular potassium, which triggers the NLRP3-, NLRC4-, and ASC-dependent activation of capase-1. Interestingly, active caspase-1 is required for activation of sterol regulatory element binding proteins (SREBPs), which are transcription factors that function predominantly in cholesterol and fatty acid biogenesis. Inactive SREBPs are located in the ER membrane. Upon cholesterol depletion, which induces their activation,

SREBPs move to the Golgi apparatus, where they are processed and liberated by two Golgi proteases. Eventually, SREBPs translocate to the nucleus, where they induce the expression of target genes and switch on lipid metabolic pathways [39,40]. SREBP activation is blocked by inhibition of caspase-1 activity or by knockdown of caspase-1, NLRP3, NLRC4, and ASC expressions. This cross-talk between active caspase-1 and SREBP is demonstrating an unexpected role of caspase-1 in cell survival, due to the repair of the damaged plasma membrane through the SREBP-dependent synthesis of lipids. However, the molecular mechanism of how caspase-1 activates SREBPs is completely unknown; this process suggests the existence of an unknown substrate of this protease, which, in turn, activates SREBPs. It would be interesting to know whether caspase-1 is also able to increase survival in macrophages, which are the primary cells involved in defense against pathogens [40]. The relevance of these less-known pathways with *P. aeruginosa* infection remains to be determined. In order to demonstrate caspase-1 involvement in disease processes, caspase-1 inhibitors are commonly used to investigate inflammasome-forming NLRs' activities [41-49], which are discussed elsewhere [50-52].

1.2.3.2 Release of Inflammatory Cytokines: IL-1β and IL-18

IL-1β is a well-known player in the process of inflammation [53-56]. Fundamentally, production of active IL-1β requires two signals; first, usually due to PRR signaling (e.g., TLR), induces pro-IL-1β expression. The second signal involves activation of inflammasome-forming NLRs and subsequently active caspase-1 [57]. Upon activation, active IL-1β is released from the cytosol, and it acts as a signaling molecule by autocrine and paracrine fashion through binding to IL-1R; this leads to production of pro-IL-18 [58], tumor necrosis factor (TNF) [59], and further synthesis of IL-1β [60].

Caspase-1 is described as the principal activator of pro-IL-1β [57,61,62] and therefore, a central regulator of the inflammatory response. In addition, active caspase-1 can also activate pro-IL-18 [63].

The active form of IL-18 is a potent stimulator of interferon gamma (IFN-γ) synthesis by NK (natural killer), Th1 (T helper), and Tc (T cytotoxic) cells. IL-18 is also involved in activation of TLR2 [64], and induction of IL-6 [65]. Interestingly, pro-IL-1α, pro-IL-1β, and pro-IL-18 lack a signal peptide for protein secretion and are released from cells by a poorly understood pathway termed unconventional protein secretion, which occurs independently of the classical ER/Golgi pathway [30,53].

1.2.3.3 Induce Inflammatory Cell Death: Pyroptosis

When a cell dies from a physiological reason such as aging, a process of normal cell turnover, the cell is cleared in the apoptotic process and inflammatory response is not activated. However, in the case of pathological cell death, production of chemokines, transmigration of leukocytes (neutrophils) to the site of damage, and activation of inflammatory response occur [66]. Therefore, cell death can be classified to different types, such as: apoptosis, necrosis, and pyroptosis [67-70], according to the Nomenclature Committee on Cell Death (NCCD) 2012 [67]. Since these subroutines are numerous and often overlap, only pyroptosis, one type of inflammatory cell death, will be focused on in this review.

Pyroptosis is a caspase-1-dependent programmed inflammatory cell death and can be distinguished clearly from apoptosis [61,62,71-73], which involves DNA fragmentation; however, it does not lead to membrane blebbing, cytochrome c release, and caspase-3 activation as observed in apoptosis. Furthermore, pyroptosis and necrosis

lead to cell swelling, pore formation, and cell lysis, which subsequently release active caspase-1 (an important characteristic that is not related to necrosis). Therefore, pyroptosis is associated with anti-pathogen response during inflammation and has characteristics of both apoptosis and necrosis [74,75]. Activation of inflammasome-forming NLRs with consequent induction of pyroptosis, has been demonstrated for several microbial pathogens [70,76-78]. In the case of bacterial pathogens, pyroptosis is a mechanism that effectively contributes to infection control; thus, some bacteria and viruses use diverse strategies to evade recognition and inflammasome-forming NLRs activation, including *P. aeruginosa* [71,79,80]. However, the molecular mechanisms of inflammasome-forming NLRs inhibition by pathogens, particularly *P. aeruginosa*, remain largely unknown. Pyroptosis was first described in mouse macrophages infected with *Salmonella typhimurium* [38] and caspase-1 induction occurs via NLRC4 and ASC [81]. However, pyroptosis induced by *Bacillus anthracis* seems not to involve NLRC4, but instead involves NLRP1 protein [82]. Interestingly, LPS-stimulated human macrophages have been shown to undergo ASC- and caspase-1-dependent pyroptosis [83]; thus, distinct models of pyroptosis exist uncertain in literature. Studies discussing the mechanism of pyroptosis inhibition by some bacterial pathogens and, in case of *P. aeruginosa*, T3SS (exoenzyme S (ExoS) and ExoU) has been suggested in the involvement of pyroptosis inhibition [84]. Indeed, the theory of induction of pyroptosis or NLR inhibition with *P. aerguionsa* infection is still undefined.

Recent studies discussed the activation of specific caspase family members (Caspase-1, -4, -5, and -11) with cystolic LPS, as well as activation of NLRP1, NLRP3, NAIP, and NLRC4 induce the cleavage of Gasdermin D (GSDMD), which is considered

to be involved in epithelial cell proliferation and causing pore-formation on cell membrane [85]. It is also reported that GSDMD pores required for IL-1β release with macrophages [86].

1.2.4 Recognition of *P. aeruginosa* by NLRs

1.2.4.1 Inflammasome-dependent Caspase-1 Activation in *P. aeruginosa*-infected Cells

A number of studies demonstrated the activation of caspase-1 with *P. aeruginosa* infection, either directly by addressing its involvement, or indirectly through studying NLRs activation and the release of pro-inflammatory cytokines active IL-1β and IL-18. It has been reported that pro-caspase-1 expressed in various human tissues with different levels of expression, as well as in some cultured cell lines [87]. It has been found that caspase-1 activation, together with IL-1β secretion, in infected human macrophages with *P. aeruginosa* up-regulated in a time- and dose-dependent [88]. Activation of single NLR led to formation of inflammasome and followed by caspase-1 activation; however, there are many NLRs that could be triggered by different activators in a timely manner and all eventually activate caspase-1. Accordingly, it should be noted there is a difference at the level of inflammatory cytokines release between inducing single NLR with well-known related activator compared to using multiple activators, which may be triggering more than a single NLR (e.g., using whole bacteria such as *P. aeruginosa*). Hence, it is worth to raise some questions regarding this: Does each NLR have limited threshold for activating caspase-1? Or, Does the activated caspase-1 have threshold with each specific activated NLR? While there is synergy between activated NLR and activation of caspase-1 and IL-1β production, why does not all of pro-caspase-1 get activated?

Some of active caspase-1 has been reported that it released extracellular. However, there is no data regarding whether there is a specific receptor on cell membrane for active caspase-1 or even its role outside of the cells. Recent studies found that overexpression of caspase-1 in infected-THP-1 cells with *P. aeruginosa* enhanced autophagy in infected cells by the increased expression of LC3-II protein, which is recruited to autophagosomal membranes [88]. Release of pro-inflammatory cytokines and induction of pyroptosis are both dependent on caspase-1 activation. Based on the literature, activation of caspase-1 is involved in the pathogenesis of *P. aeruginosa* and contributes to an efficient immune response by the host. However, the role of caspase-1 with either cell death or other less-known pathways in *P. aeruginosa* infection still remains elusive.

1.2.4.2 Importance of NOD1 in Host Defense Mechanisms against *P. aeruginosa*

Some studies have highlighted the importance of NOD1 and NOD2 in innate immune response and, as a result, those have become well-characterized members in the NLR family [12]. NOD1 is expressed ubiquitously in various cell types, while NOD2 is primarily found in antigen presenting cells such as macrophages and dendritic cells (DCs) [89]. Furthermore, NOD1 and NOD2 are cystolic receptors for specific muropeptides present in bacterial peptidoglycan to achieve this sensing [90]. Where NOD1 recognizes diaminopimelic acid (DAP) found in the peptidoglycan of many Gram-negative bacteria like *P. aeruginosa*, NOD2 is a more general sensor of bacteria and detects muramyl dipeptide (MDP) present in the peptidoglycan of both Gram-positive and -negative bacteria. In bacteria unable to enter the cell, the mechanism by which these peptides are detected remains unknown [90-92]. Most NLRs do not alter NF-κB signaling, however, NOD1 and NOD2 are associated with NF-κB activation after stimulation with

peptidoglycan [90,93-95]. Studies have reported the internalization of *P. aeruginosa* within human epithelial cells [96,97], and investigated the role NOD1 with *P. aeruginosa* peptidoglycan, which involved in NF-κB activation and bacterial killing [21]. Another study identified a novel mechanism of *P. aeruginosa* to deliver peptidoglycan to NOD1 in host cells via outer membrane vesicles (OMVs) [98]. While it has been demonstrated that the involvement of NOD1 and human β-defensin 2, which is a pulmonary antimicrobial peptide [99], in direct killing of Gram-negative bacteria, *Helicobacter pylori* [100], it will be interesting to look at mechanisms involved in NOD1-dependent intracellular bacterial killing, as well induction of autophagy [101] and host defense to elucidate the role of NOD1 in *P. aeruginosa* infection.

1.2.4.3 NLRC4 and TLR5 have unique and redundant roles in lung immunity against *P. aeruginosa*

NLRC4 is required for the activation of caspase-1 in macrophages infected with pathogenic bacteria, including *Salmonella enterica* [102,103], *Legionella pneumophila* [104-106], and *P. aeruginosa* [107,108]. The activation of caspase-1 by these pathogenic bacteria requires a functional bacterial secretion system, which has been suggested as a link between bacterial pathogenicity and NLRC4 activation [109]. These secretion systems, which include the T3SS and T4SS, act as molecular needle-like structures that inject effector proteins into the cytosol of host cells and are critical for pathogen colonization. Flagellin, the principle substituent of the flagellum, is also important for activation of the NLRC4 inflammasome [102,103]. Since the delivery of purified flagellin to the macrophage cytosol triggers caspase-1 activation through NLRC4 [102,103], it had been thought that NLRC4 is activated in macrophages via the leakage of

small amounts of flagellin through a T3SS (for example, *S. enterica* and *P. aeruginosa*), or T4SS (for example, *L. pneumophila*), during infection [110]. However, *Shigella flexneri*, an aflagellated pathogenic bacterium, also induces activation of the NLRC4 inflammasome through the T3SS [81]. Furthermore, flagellin-deficient *S. enterica* and *P. aeruginosa* can activate NLRC4 at high ratios of bacteria to macrophages, which further suggest that factors other than flagellin can induce activation of the NLRC4 inflammasome [111,112]. Initial insights into the flagellin-independent pathway were provided by the observation that proteins, which form the basal body rod component of the T3SS, such as PrgJ, can activate the NLRC4 inflammasome. PrgJ-like proteins contain regions structurally homologous to the carboxy-terminal portion of flagellin [111], which is the critical portion of flagellin that is sufficient to trigger NLRC4 inflammasome activation [103,113].

The NLRC4 inflammasome has been recently identified as an essential element in innate immunity against *P. aeruginosa* [107,114], which is activated by flagellin and T3SS rod protein [111,115]. Although *P. aeruginosa* strains express multiple cell-associated and secreted virulence factors, which activate innate immune responses, their specific role in inflammasome activation remains elusive. Indeed, while activation of the NLRC4 inflammasome depends on flagellin and the T3SS [114], flagella-deficient *P. aeruginosa* strains are still able to induce the inflammasome activation. Other data suggests that *P. aeruginosa* pilin and the *rhs* gene product can activate inflammasome-forming NLRs, although the underlying mechanisms are unknown [116,117]. Remarkably, study findings indicated that redundant and cooperative interactions between TLR5 and NLRC4 in lung immunity against *P. aeruginosa* infection in mice

model and double-knockout mice with those PPRs resulted in an impaired bacterial clearance [118]. It is noteworthy to compare these finding with flagella-deficient *P. aeruginosa* strain as other studies explored the role of TLR4 and TLR5 in the host response to pulmonary infection with *P. aeruginosa* [119,120].

1.2.4.4 NLRP3 is essential for autophagy not IL-1β production

The NLRP3 is the most important type of NLRs as it is activated by numerous PAMPs and DAMPs [73]. Inflammasome-forming NLRP3 is activated by a plethora of microbial stimuli [15,29,121-123], as well endogenous stimuli such as uric acid, cholesterol or hydroxyapatite crystals, silica, aluminum salts, asbestos, malarial hemozoin, amyloid deposits, and fatty acids [28]. Given the chemical and structural diversity of the NLRP3 activators, it has been hypothesized that NLRP3 does not interact directly with its activators; instead, its activation is triggered through an intermediate cellular signal elicited by all these stimuli. Studies have reported that the transcription factor NF-κB, TNF-α, IL-1β, and SYK (a non-receptor tyrosine kinase) are effective in inducing NLRP3 expression and promoting caspase-1 activation in response to NLRP3 activators [124-128]. Several theories have been proposed for the identity of the cellular signal responsible for NLRP3 activation, including a change in the intracellular concentration of K+ and Na+, the formation of a large pore in cell membrane, the release of cathepsins from damaged lysosomes, the production of ROS, and damage in the mitochondria. [108,129-131]. Therefore, further studies are required to clarify the detailed mechanisms linking numerous chemically and structurally diverse stimuli from microbial pathogens as activators of the inflammasome-forming NLRP3. It is worth to mention, as it has been reported that SYK phosphorylation is involved in NLRP3-mediated caspase-1 activation

[130], that in our previous work we found that pre-treatment of human macrophages with SYK inhibitor R406 resulted in a significant inhibition of SYK phosphorylation and down-regulation of IL-1β production in infected cells with *P. aeruginosa* [132]

The production of ROS and the release of mitochondrial DNA have been suggested to act as a common cellular signal upstream of NLRP3. However, ROS scavengers and NADPH-oxidase inhibitors block activation of NLRP3 [133-135]. Indeed, NLRP3 has been proposed to integrate signals that indicate cellular damage or stress [6,134,136]. *P. aeruginosa* can potentially activate NLRP3 as the pathogen is able to induce ROS production via several mechanisms, (for example, via the cytotoxin pyocyanin), which can oxidize glutathione and inactivate catalase contributing to the oxidative stress mediated cell damage [137,138]. However, mutant strains of *P. aeruginosa* exhibited a reduced ability to cause intracellular ROS production in infected human cells [97,139]. Accordingly, these findings inspire further studies on the role of specific *P. aeruginosa* virulence factors in the NLRP3 inflammasome activation.

Remarkably, Chen *et. al.* studied partial knockdown of NLRP3, which decreased caspase-1 activation and significantly reduced pore formation in macrophages, whereas IL-1 β release was not significantly impaired [140]. This study supported that NLRP3 is not essential for IL-1β release. However, the low levels of capsase-1 from partial knockdown of NLRP3 might be sufficient for maturation of IL-1β, or other inflammasome-forming NLR may also contribute to IL-1β production. Recent studies demonstrated the activation of NLRP3 in human macrophages with *P. aeruginosa*, and explored a novel mechanism by this bacterial pathogen to escape from macrophage

intracellular killing by activation of NLRP3, as well as trigger the formation of autophagosome following NLRP3 overexpression [88].

1.2.5 Negative regulatory role of NLRs

Most NLRs induce inflammatory responses, however, emerging studies of knockout gene(s) model have revealed that a number of NLRs negatively regulate inflammatory responses including: NLRC3, NLRC5, NLRP4, NLRP6, NLRP10, NLRP12, and NLRX1 [141-153]. This unique mechanism employed by members of this novel family of PRRs to regulate the host immune response following pathogen exposure is worth to be further addressed with *P. aeruginosa* infection, particularly NLRC3 and NLRC5, which will significantly improve our overall understanding of host innate immunity against *P. aeruginosa* infection.

1.2.5.1 NLRC3 promotes host resistance

NLRC3 is one of the most regulatory characterized NLRs, which attenuates T-cell signaling via TCR and co-stimulatory molecules, and alters NF-κB activation through interaction with TRAF6 [154]. It has been demonstrated that TRAF6 is involved in the TLR-mediated response to *P. aeruginosa* in human airway epithelial cells [155]. Remarkably, studies showed that NLRC3 down-regulated NF-κB transcriptional activity, which is induced either by NOD1 or NOD2 receptors, without any effects on the expression of these receptors [156]. Another pathway for NLRC3 as a negative regulator, is the interaction with a trans-membrane protein called stimulator of IFN genes (STING), which functions as an essential signaling adaptor in linking the cytosolic detection of DNA to TANK binding kinase 1 (TBK1) [148,152,157-159]. The latter results in IFN-regulatory factor 3 (IRF3) activation and pro-inflammatory cytokine production

[160,161]. Interestingly, IRF3 contributes to host response during *P. aeruginosa* lung infection [162]. Recent studies identified that overexpression of a regulatory NLRC3 significantly attenuated disease progression, decreased the production of cytokines release, and promoted degradation of interleukin-1 receptor-associated kinase 1 (IRAK1) after *P. aeruginosa* infection in keratitis model [151]. Although NLRC3 has no effect on both NOD1 and NOD2 protein levels, study findings indicated that NLRC3 suppressed NF-κB transcriptional activity in human embryonic kidney cells, while NOD1 and NOD2 induced NF-κB activation [156].

1.2.5.2 NLRC5 interacts with NLRP3 and negatively regulates NOD1 and NOD2

Human NLR family members are typically localized in the cytoplasm. However, NLRC5 and CIITA (NLRA) can be found in the nucleus [7], which are regulating the gene transcription of major histocompatibility complex (MHC) class I and class II, respectively [163]. NLRC5 is the largest member of NLRs that also contains the largest number of C-terminal LRRs [164]. NLRC5 negatively regulates inflammatory responses through down-regulation of NOD1 and NOD2, which both are induced NF-κB transcriptional activity [42,144,165]. However, the complexity associated with NLRC5 has been addressed in a study related to the role of NLRC5 in inflammasome activation in human monocytic cells infected with a panel of bacteria [166]. The findings indicated that NLRC5 cooperated with NLRP3, but not other NLR, to induce inflammasome activation.

1.2.6 Conclusion

Literature related to NLRs has provided evidence that several members of the NLR family play important roles in an inflammasome formation and more beyond that.

Through model studies, NLRs can be suitably stimulated to provide adequate immune responses, confirming that NLRs can be an axis to achieve immunity. Yet, respiratory tracts in immunocompetent individuals, innate mechanisms are sufficient to manage infection by *P. aeruginosa*. These mechanisms become disabled in the CF patient, which is permitting chronic infection. As the bacteria mutate and adapt, NLR stimulation may alter and even be exaggerated, which further promotes inflammation as failing lung function seen in these patients. This paradigm involving the relationship between the evolving bacterial adaptations and infected CF, where manipulating the NLR response, may prove to be beneficial.

A major advance in our understanding of infection and immunity occurred with the discovery of NLRs. These intracellular PRRs are able to sense a variety of bacterial products and aid in initiation of an appropriate inflammatory response [21]. NLRs enable the host immune system to recognize and respond to microbes by their PAMPs and trigger the earliest immune responses that lead to inflammation. Microbial agents, or their PAMPs, via their interaction with NLRs and other pattern recognition receptors (PRRs), may be critically important in the pathogenesis of inflammatory lung diseases. A better understanding of these mechanisms is of fundamental importance to expand our knowledge about *P. aeruginosa* infection and innate immunity, as it may identify potentially new therapeutic target(s) for treating the threatening inflammatory lung diseases. This area of research is still in infancy. More knowledge of the NLR signaling pathways, as well as increasing evidence for the role of NLR ligands in the molecular pathogenesis of diseases, will be needed for the development of new therapeutic strategies, especially for infectious lung diseases. Understanding the complex

mechanisms underlying NLR localization and function will provide additional data that might help devise novel therapeutic approaches involving NLRs and their agonists, in an attempt to attenuate the immune responses associated with *P. aeruginosa* infection.

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Table

Table 1 NLR Family Members according to their domain organization

Protein Name	Synonym	Gene Size (bp)	Gene Accession #	Location (chromosome)	Protein Accession #	Protein Size (a.a)	Domain Type	NLR Family	Domain Structure
NOD1	NLRC1; CARD4; CLR7.1	54251	AF126484	7p15-p14	NP_006083	953	CARD	NLRC	CARD-NACHT-NAD-LRR
NOD2	CD; BLAU; IBD1; NLRC2; NOD2B; CARD15; CLR16.3; PSORAS1	35938	AF178930	16q21	NP_071445	1040			CARD2x-NACHT-NAD-LRR
CIITA	NLRA; MHC2TA; C2TA	500	AF000002	16p13	AAB92362.1	94		NLRA	(CARD)-AD-NACHT-NAD-LRR
NLRC3	NOD3; CLR16.2	38360	BK001112	16p13.3	NP_849172	1065	Untypical CARD	NLRC	Card?-NACHT-NAD-LRR
NLRC4	CLAN; IPAF; CLAN1; CLANA; CLANB; CLANC; CLAND; CADR12; CLR2.1	41295	AF376061	2p22-p21	NP_067032	1024			Card?-NACHT-NAD-LRR
NLRC5	NOD4, NOD27, CLR16.1	66451	AF389420	16q13	NP_115582	1866			Card?-NACHT-NAD-LRR
NLRP1	NAC; CARD7; NALP1; SLEV1; DEFFCAP; PP1044; VAMAS1; CLR17.1; DEFCAP-L/S	83114	AB023143	17p13.2	NP_127497	1399	PYRIN	NLRP	PYR-NACHT-NAD-LRR-FIIND-CARD
NLRP2	NBS1; PAN1; NALP2; PYPAF2; CLR19.9	35859	AK000517	19q13.42	NP_060322	1062			PYD-NACHT-NAD-LRR
NLRP3	AII; AVP; FCU; MWS; FCAS; CIAS1; NALP3; C1orf7; CLR1.1; PYPAF1; AGTAVPRL; Cryopyrin	32953	AF054176	1q44	NP_004886	1036			PYD-NACHT-NAD-LRR
NLRP4	CT58; PAN2; RNH2; NALP4; PYPAF4; CLR19.5	45278	AF479747	19q13.42	NP_604393	994			PYD-NACHT-NAD-LRR
NLRP5	MATER; NALP5; PAN11; PYPAF8; CLR19.8	62085	AY154460	19q13.42	NP_703148	1200			PYD-NACHT-NAD-LRR
NLRP6	AVR; NAVR; PAN3; NALP6; PYPAF5; CLR11.4; NAVR/AVR	6735	AF479748	11p15	NP_612202	892			PYD-NACHT-NAD-LRR
NLRP7	HYDM; PAN7; NALP7; NOD12; PYPAF3; CLR19.4	23997	AF464765	19q13.42	NP_996611	1009			PYD-NACHT-NAD-LRR
NLRP8	PAN4; NALP8; NOD16; CLR19.2	40798	AY154463	19q13.42	NP_789781	1048			PYD-NACHT-NAD-LRR
NLRP9	NOD6; NALP9; PAN12; CLR19.1	29971	AY154464	19q13.42	NP_789790	991			PYD-NACHT-NAD-LRR
NLRP10	NOD8; PAN5; PYNOD; NALP10; CLR11.1	3904	AY154465	11p15.4	NP_789791	655			PYD-NACHT-NAD
NLRP11	NOD17; PAN10; NALP11; PYPAF6; PYPAF7; CLR19.6	51366	AY095145	19q13.42	NP_659444	1033			PYD-NACHT-NAD-LRR
NLRP12	RNO; PAN6; RNO2; FCAS2; NALP12; PYPAF7; CLR19.3; Monarch1	30811	AY095146	19q13.41	NP_653288	1016			PYD-NACHT-NAD-LRR
NLRP13	NOD14; PAN13; NALP13; CLR19.7	36392	AY154468	19q13.42	NP_789780	1043			PYD-NACHT-NAD-LRR
NLRP14	NOD5; PAN8; GC-LRR; NALP14; CLR11.2	51058	BK001107	11p15.4	NP_789792	1093			PYD-NACHT-NAD-LRR
NAIP	BIRC1; NLRB1; psiNAIP; CLR5.1	56632	4671	5q13.1	NP_004527	1241	BIR	NLRB	BIR3x-NACHT-LRR
NLRX1	NOD5; NOD9; NOD26; DLNB26; CLR11.3	15286	AB094095	11q23.3	NP_078894	921	Undefined	NLRX	X-NACHT-NAD-LRR

AD, acidic activation domain; **CARD**, caspase activating and recruitment domain; **LRR**, leucine-rich repeat; **BIR**, baculovirus inhibitor of apoptosis repeat; **PYD**, pyrin domain; and **NAD**, NACHT-associated domain.

Figure Legend

Figure 1 Human NLRs based on their functions.

Members of NLR family play a critical role in the regulation of innate immune response and have diverse functions, which can be divided into four broad categories; nodosome, regulatory inflammasome, transcriptional activator NLR, and reproductive and embryogenesis NLR. Most NLR functions range from pathogen and damage sensing to antigen presentation, as well as, suppression of inflammation. As non-immune pathways, some NLRs are potentially involved in embryonic development.

Figure 2 Inflammasome-forming NLR regulates inflammation during infection and tissue injury.

NLRs are activated by a wide array of either PAMPs or DAMPs. The initial event leads to assembling of inflammasome, followed by activation of caspase-1 and release of IL-1β as well as IL-18. Release of IL-1β results in recruitment of effector cell populations of the immune response and tissue repair. Several inflammasome inhibitors, either endogenous or exogenous, have been shown to inhibit the activity and the formation of inflammasome. **Ac-YVAD-cmk**; acetyl-tyrosyl-valyl-alanyl-aspartylchloromethylketone, **AG126**; tyrosine kinase inhibitor, **COPs**; CARD-only proteins, **ICEBERG**; CARD-containing proteins, **INCA**; inhibitory caspase recruitment domain, **POP**; Pyrin-only protein, **XIAP**; X-linked inhibitor of apoptosis, **Z-VAD-fmk**; Nbenzyloxycarbonyl- Val-Ala-Asp-fluoromethylketone.

Figure 3 Multiple biological responses following caspase-1 activation.

Different inflammasome-forming NLRs activate caspase-1, which cleaves pro-IL-1β and pro-IL-18 to mature biologically active forms. Following caspase-1 activation, there is increasing evidence that caspase-1 contributes to regulation of other several pathways. Dash line shows tentative pathways.

Figures

Figure 1

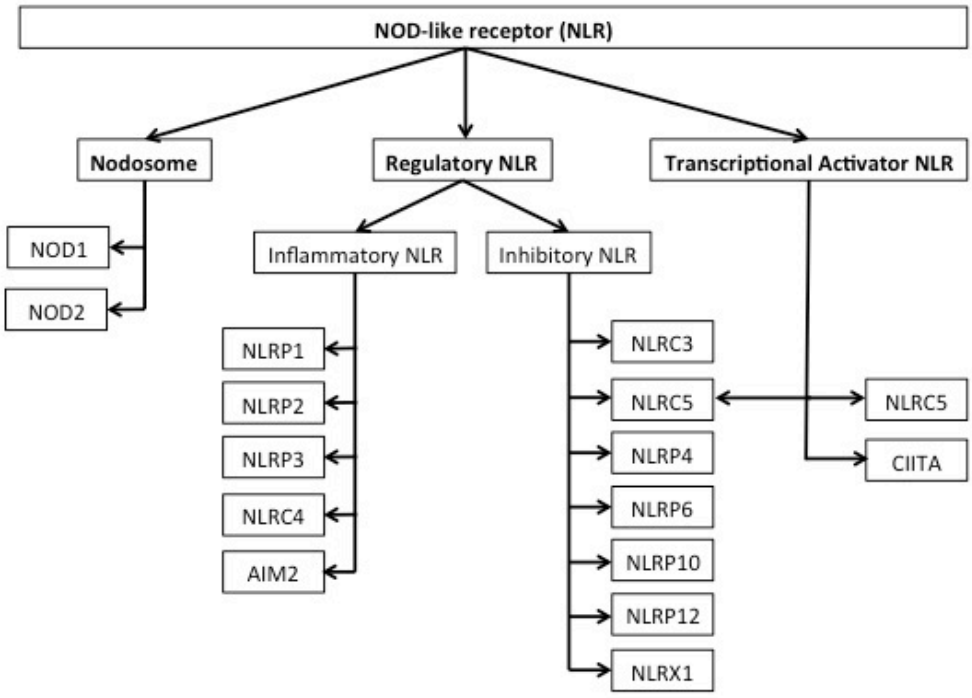


Figure 2

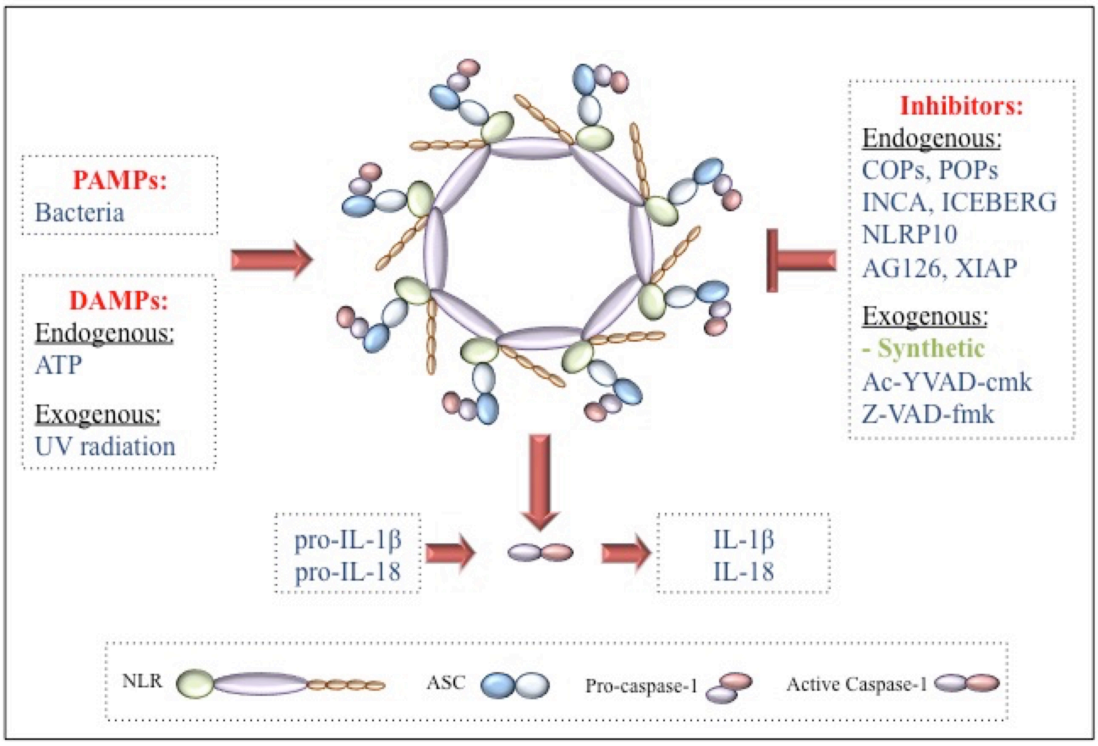
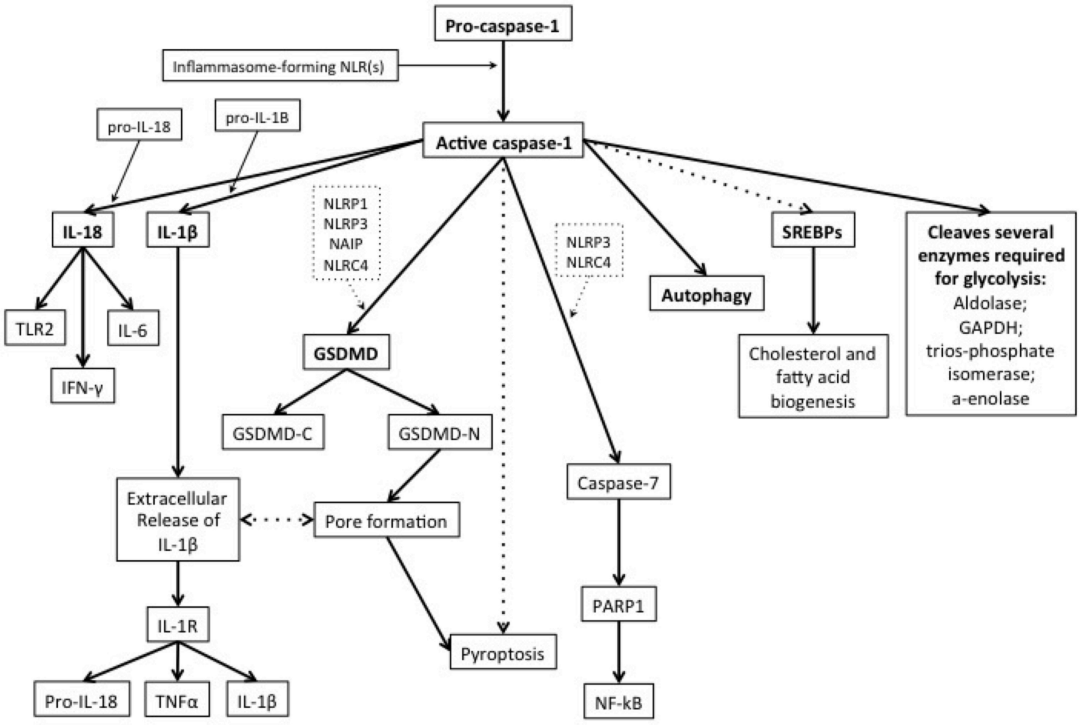

PAMPs:
Bacteria
DAMPs:
Endogenous:
ATP
Exogenous:
UV radiation
Inhibitors:
Endogenous:
COPs, POPs
INCA, ICEBERG
NLRP10
AG126, XIAP
Exogenous:
- Synthetic
Ac-YVAD-cmk
Z-VAD-fmk
pro-IL-1β
pro-IL-18
IL-1β
IL-18
NLR
ASC
Pro-caspase-1
Active Caspase-1

Figure 3


Pro-caspase-1
Inflammasome-forming NLR(s)
pro-IL-18
pro-IL-1B
Active caspase-1
IL-18
IL-1β
NLRP1
NLRP3
NAIP
NLRC4
NLRP3
NLRC4
Autophagy
SREBPs
Cleaves several enzymes required for glycolysis:
Aldolase;
GAPDH;
trios-phosphate isomerase;
a-enolase
TLR2
IL-6
IFN-γ
GSDMD
Cholesterol and fatty acid biogenesis
GSDMD-C
GSDMD-N
Caspase-7
Extracellular Release of IL-1β
Pore formation
PARP1
IL-1R
Pyroptosis
Pro-IL-18
TNFα
IL-1β
NF-kB

1.3 SYK Tyrosine Kinase as Target Therapy for *Pseudomonas aeruginosa* Infection

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Abstract

Spleen tyrosine kinase (SYK) is a non-receptor tyrosine kinase, which associates directly with extracellular receptors and is critically involved in signal transduction pathways in a variety of cell types for the regulation of cellular responses. SYK is expressed ubiquitously in immune and non-immune cells, and has a much wider biological role than previously recognized. Several studies have highlighted SYK as a key player in the pathogenesis of a multitude of diseases. *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen, which is responsible for systemic infections in immunocompromised individuals and accounts for major cause of severe chronic lung infection in cystic fibrosis patients, subsequently resulting in progressive deterioration of lung function. Inhibition of SYK activity was explored as a therapeutic option in several allergic disorders, autoimmune diseases, and malignancies. This review focuses on SYK as therapeutic target and describes the possibility of how current knowledge could be translated for therapeutic purposes, to regulate immune response to the opportunistic pathogen *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, Infection, Cystic fibrosis, Inflammation, SYK, small molecule inhibitor, CFTR

1.3.1 Introduction

Spleen tyrosine kinase (SYK) is a non-receptor tyrosine kinase involved in signal transduction in a variety of cell types; it associates with different receptors on the surface of various cells, such as B cells, mast cells, monocytes, macrophages, and neutrophils and even osteoclasts and breast cancer cells. Following the engagement of these receptors with their ligands, SYK is activated and orchestrates diverse cellular responses, including cytokine production (in T cells and monocytes) and phagocytosis (in macrophages) [1,2]. SYK is expressed ubiquitously in both hematopoietic [3-14] and non-hematopoietic cells [15-20]. Notably, this widespread expression of SYK in human tissues implies that it plays important roles in different organs. Importantly, SYK is expressed in lung epithelial cells [21,22], which are the major components of the airway lining and the sites of infection by *Pseudomonas aeruginosa*. The role of SYK in these structural cells is puzzling, but recent studies shed some light on it. For these reasons, it may represent an attractive target for new therapeutics strategy of treating *P. aeruginosa* infection using inhibition of SYK kinase. In this review, the role of SYK and the effect of SYK inhibitor in treatment of *P. aeruginosa* infection are discussed.

1.3.2 Structural Basis of SYK Activation

SYK, a 72 kDa protein, is composed of two SRC homology (SH2) and one kinase domains, with an interdomain A located between the two SH2 domains and interdomain B located between the SH2 and kinase domains; the interdomains contain linker tyrosines, which can undergo phosphorylation (Figure 1) [22-25]. SYK contains at least ten tyrosine residues that can be autophosphorylated and thus provide binding sites for other molecules bearing SH2 domains [26]. Due to its catalytic activity and the ability to bind

other proteins via the interaction between phosphorylated tyrosines and SH2 domains, SYK has both kinase and adaptor protein properties.

There are three states of SYK: inhibition of the kinase, activated kinase via phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) and activated kinase via phosphorylation of linker tyrosines. In the inhibited kinase state, the binding occurs between interdomain A, interdomain B, and the kinase domain, producing the stable configuration of SYK; breaking apart this arrangement will allow for the activation of the protein kinase to occur [24]. ITAM tyrosine residues are rapidly phosphorylated following classical immunoreceptors' engagement, i.e. B cell receptors (BCRs), T cell receptors (TCRs), and Fc receptors (FcRs), leading to the recruitment and activation of SYK. The other state of SYK is the activation of the kinase through autophosphorylation of the linker tyrosines in the interdomains; this process does not involve the dependence on the phosphorylated ITAMs for activation [22-25]. SYK can sustain activation following the temporary interaction with phosphorylated ITAMs by means of autophosphorylation of the linker tyrosines [24].

SYK activation is not restricted to the two mechanisms stated; studies have also shown that SYK mediates signaling by classes of receptors, including integrin, G-protein coupled, and C-type lectins that do not contain conventional ITAMs [22,27]. During an inflammatory response of the immune cells, as a result of a variety of different signaling pathways, cytokines are produced as well; studies have shown that cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, produced during inflammation also have the ability to activate SYK by means of cytokine signaling [27].

Collectively, these studies have dramatically changed our view of the SYK tyrosine kinase.

1.3.3 SYK and innate immunity

Innate immune system plays a leading role through the cooperation of different germline-encoded pattern recognition receptors (PRRs) to detect both pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) and trigger immune responses. Studies have shown that many PPRs participate in the immune response to *P. aeruginosa* infection, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), etc. [28,29]. Recently, SYK has been found to be a vital component of these pathways, which plays a crucial role in the innate immune response including pathogen recognition, inflammasome activation and even anti-fungal defense [24,30,31]. Following the activation of the kinase, SYK-mediated downstream signaling occurs as a result. SYK can bind directly to four binding partners: VAV, phospholipase Cγ (PLCγ), phosphoinositide 3-kinase (PI3K) and SH2 domain of the leukocyte protein 76 or 65 (SLP76 or SLP65, respectively). These four binding partners will further activate downstream signaling components that will lead to the eventual change in cellular response. Such cellular responses include reactive oxygen species (ROS) production, proliferation of cells, cytokine release, and inflammatory responses [24]. Up to now, there is little research on the involvement of SYK in cellular responses to *P. aeruginosa* infection, and targeting SYK for protecting infected human cells against the deleterious effects associated with this infection. However, it has been better demonstrated in several allergic disorders, autoimmune diseases, malignancies, and innate antifungal immunity.

It is well established that SYK activation in leukocytes is essential for phagocytosis and the development of B- and T-lymphocytes [24]. Studies have shown that many CLRs, such as Dectin-1 (also known as CleC7A) and Mincle (also known as CleC4e), resist the fungi mainly by activating the downstream SYK-caspase recruitment domain-containing protein 9 (CARD9)-nuclear factor kappa B (NF-κB) signaling pathway [32-36]. Recent studies have revealed the importance of SYK during fungal infection *Aspergillus fumigatus* [37]. Researchers have proved that SYK associates with the invasive breast cancer [38] and SYK is closely related to the occurrence and development of digestive tract tumors [39].

Besides, because SYK is positioned upstream in the cell-signaling pathway, therapies targeting SYK might be more advantageous than inhibiting a single downstream event [40]. These make SYK a therapeutic target for an array of inflammatory diseases and for this reason, many pharmaceutical companies and academic institutions have been involved in the development of SYK small-molecule inhibitors. Recent studies have demonstrated the ability of SYK to regulate production of pro-inflammatory molecules by bronchial epithelial and monocytic cells, which are stimulated with TNF- α, rhinovirus, or *P. aeruginosa* [25,27,30,31,41]. For these reasons, it may represent an attractive target for new therapeutics strategy of treating *P. aeruginosa* infection using inhibition of SYK kinase. Indeed, several studies have highlighted SYK as a key player in the pathogenesis of multitude of diseases [2,42-51]. Several pathologies can be treated through the inhibition of SYK activity. Indeed, more selective commercially available small molecule SYK inhibitors show the great interest in this field [52].

1.3.4 SYK and cystic fibrosis

CF is an autosomal-recessive disease, mainly occurring in the Caucasian population. The condition is the manifestation of mutations in a transmembrane protein, called cystic fibrosis transmembrane conductance regulator (CFTR), which commonly results in a loss of the protein or deficiency of its function [53,54]. Mostly, CFTR functions as chloride ion (Cl^-) channel at the apical surface of secretory epithelia. CFTR is a member of the ATP-Binding Cassette transporter family, which hydrolyzes ATP to pump substrates, such as ions, vitamins, drugs, toxins, and peptides across biological membranes [55]. Since its discovery in 1989, many mutations in the gene have been identified; approximately 127 are confirmed as CF disease-causing [56]. Among these mutations, a phenylalanine (3-bp) deletion at position 508 in the polypeptide chain (ΔF508) is resulting in a protein that fails to mature properly and becomes degraded [55,57]. ΔF508 is present in nearly 85% of CF patients in at least one allele. A connection has been made between mutant or missing CFTR in human lung epithelial cell membranes and a failure in innate immunity, which is leading to initiation of *P. aeruginosa* infection. Epithelial cells use CFTR as a receptor for internalization of *P. aeruginosa* via endocytosis and subsequent removal of bacteria from the airway that does not occur in the absence of functional CFTR and results in increased bacterial loads in the lungs [58]. The static mucosal environment is presumed to render individuals susceptible to opportunistic infections, and CF patients become infected, to some extent in an age-related pattern, by multiple microorganisms but particularly, *Haemophilus influenzae*, *Staphylococcus aureus*, the *Burkholderia cepacia* complex, and ultimately, a high proportion (as many as 80% of adult CF patients) are infected with *P. aeruginosa* [59]. *P. aeruginosa* becomes a

challenge to treat, as a result of its physiological properties, pattern of gene expression and antibiotic resistance, and because of this growing in biofilms, which is significantly different from planktonic cultures [60,61]. This persistent bacterial infection underlies the chronic lung inflammation that CF patient experience. Understanding the changes in lung innate immune mechanisms as a result of dysfunctional CFTR and the persistent *P. aeruginosa* infection is paramount to changing the natural course of this disease.

The number of CFTR protein copies on the plasma membrane results from a balance between anterograde trafficking (i.e., CFTR is delivered from the endoplasmic reticulum to the plasma membrane), endocytosis (a process through which CFTR is retrieved from the membrane into vesicles), and recycling (with return of the internalized CFTR to the plasma membrane). Remarkably, one of the protein kinases that is involved in CFTR trafficking is SYK. This non-receptor tyrosine kinase has been reported to phosphorylate CFTR leading to decreased levels of CFTR in the plasma membrane [62,63]. Such a role of SYK in regulating protein trafficking has been reported previously for other substrates, for examples: trafficking a resident of the trans-Golgi network (TGN) 38 [64], trafficking of engaged high affinity IgE receptor (FcεRI) [65], and the small GTPase Rac1 [66], the latter was shown to play a role in CFTR trafficking and membrane anchoring [67]. Recent findings have shown that phosphorylation of CFTR by SYK resulted in reducing the CFTR plasma membrane abundance [68]. Accordingly, SYK inhibition may stabilize the plasma membrane level of CFTR. SYK knockdown in airway epithelial cells down-regulates proinflammatory mediators, such as IL-6 and ICAM-1 [22]; both are elevated in CF patients [69]. Recent studies expanded our understanding to recognize SYK as a potential target to stabilize CFTR plasma

membrane level and attenuate the proinflammatory mediators in *P. aeruginosa*-infected CF patients.

1.3.5 Innate immune response to *Pseudomonas aeruginosa* infection

P. aeruginosa causes systemic life-threating infection in immunocompromised individuals and chronic lung infection in CF patients. The major determinant of morbidity and mortality in CF patients can be attributed to the progressive deterioration of lung function resulting from chronic infection by such a ubiquitous opportunistic pathogen as *P. aeruginosa* [30,70]. During the infectious process, *P. aeruginosa* provokes a potent inflammatory response of infected tissue characterized by the activation of transcription factors, NF-κB and activator protein 1 (AP-1). This results in the release of pro-inflammatory mediators, i.e. cytokines TNF-α, IL-1β, IL-6, chemokines IL-8 and RANTES (regulated on activation normal T cell expressed and secreted), increased expression of adhesion molecules (intercellular adhesion molecule, ICAM-1), induces the release of ROS, recruitment of activated neutrophils, and severe tissue damage, which eventually causes lung failure [71]. The infection of the airway by *P. aeruginosa* is accompanied by the activation of pro-inflammatory intracellular signaling pathways [72]. The activation of intracellular protein kinases has a significant role in the pathogenesis of *P. aeruginosa* lung infection. It has been demonstrated that both the bacterial invasion and cytotoxic effect of *P. aeruginosa*, as well as hyper-production of IL-8 and mucin by infected lung epithelial cells, depend on the activation of the p38 and ERK1/2 mitogen-activated protein kinase (MAPK) signaling cascade and Src-like tyrosine kinases p60Src, p59Fyn, and Lyn [73-76].

Airway inflammation is a dominant pathophysiological characteristic of *P.*

aeruginosa infection influencing both the severity of the disease and its outcomes. Also, *P. aeruginosa* is intrinsically resistant to many antibiotics, making treatment difficult and often unsuccessful [77]. Based on the rapidly growing understanding of intracellular signaling pathways involved in the pathogenesis of bacterial inflammation, targeting the inhibition of specific signaling pathways/molecules is a potential treatment strategy in *P. aeruginosa* lung infection.

1.3.6 Effect of SYK inhibitor in *Pseudomonas aeruginosa* infection

Potent signaling abilities of SYK are due to both its molecular structure and strategic localization in the proximal part of intracellular signaling cascades. Considering the vital role of inflammation in the pathogenesis of *P. aeruginosa* lung infection, the down-regulation of pro-inflammatory signaling pathways via a SYK inhibitor may be a beneficial addition to the antibacterial therapy of such conditions. Studies have found that natural SYK inhibitor piceatannol can inhibit the essential mechanisms of *P. aeruginosa* pathogenesis, i.e. bacterial internalization, production of pro-inflammatory mediators, oxidative stress, and apoptosis of infected human airway epithelial cells [30], which is supporting the involvement of SYK in the regulation of inflammatory responses caused by *P. aeruginosa*. Other studies using a model of human monocytic cells found that a small molecule inhibitor R406 decreased both inflammatory responses and apoptosis induced by *P. aeruginosa* infection [31]. SYK has been recently identified as a crucial mediator of NLRP3 inflammasome activation and IL-1β secretion in macrophages stimulated with fungi and crystals [78]. Although the underlying molecular mechanisms are still being defined, SYK is known to regulate ROS production and lysosomal activity, two significant signals for NLRP3 inflammasome activation in macrophages [24]. It has

recently been found that inhibition of SYK reduced the release of bioactive IL-1β by macrophage cells infected with a *P. aeruginosa*, [31] suggesting that SYK may regulate innate immune responses to *P. aeruginosa* via its involvement in inflammasome activation.

The role of SYK kinase in the regulation of inflammasome activation and ROS production induced by *P. aeruginosa* infection of human cells need to be addressed to clarify the mechanisms behind the involvement of SYK-mediated signaling in the regulation of innate immune responses to *P. aeruginosa* infection. Based on the literature, studies suggest an association of SYK with regulation of innate immune and inflammatory responses to *P. aeruginosa*; it endorses that SYK mediates inflammasome activation and promotes an enhanced production of pro-inflammatory mediators by infected cells. Indeed, a significant decrease in the release of pro-inflammatory mediators by both *P. aeruginosa*-infected human macrophage (IL-1β and TNF-α) and lung epithelial cells (TNF-α) following SYK inhibition by R406 has been reported recently [31].

1.3.7 Concluding remarks

P. aeruginosa can cause chronic lung infection and systemic life-threating diseases in cystic fibrosis patients and immunocompromised individuals. Based on recent evidence, SYK mediates innate immune response to *P. aeruginosa* infection and it can be involved in the amount and activity of CFTR protein at the plasma membrane. Also, SYK is considered as a potential target of anti-inflammatory therapy of various clinical conditions. Indeed, SYK is mostly controlling the inflammatory process and inhibition of SYK activity is a valuable strategic therapy in *P. aeruginosa* infection. While a large

number of small molecules have been synthesized and tested as SYK inhibitors, it has been reported that some unwanted side effects are associated with its application. However, the therapeutic activity of some SYK inhibitors has already been demonstrated, and they are currently in the advanced phases of clinical trials. Despite these encouraging results, some issues may relate to these molecules such as an increased probability of off-target effects. The role of SYK in cellular responses to *P. aeruginosa* in infected CF patients or animal models with deficiency in *CFTR* is completely unknown. Further research to discover capability of inhibition of SYK in CF patients and animal models to demonstrate its effect on the CFTR level along with *P. aeruginosa* infection and associated inflammatory responses, which significantly contribute to the pathogenesis of *P. aeruginosa* pulmonary infections may represent a reasonable approach.

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Figure Legend

Figure 1 Structure of SYK protein

A schematic diagram of the linear structure of SYK with the tyrosines marked that are phosphorylated after activation.

Figure 2 General mechanism of SYK activation and SYK-mediated signaling

AKT: protein kinase B; **ERK**: extracellular signal-regulated kinase; **GPCRs**: G protein-coupled receptors; **IL-1R**: interleukin-1 receptor; **JNK**: c-Jun N-terminal kinase; **PM**: plasma membrane; **TNFR**: tumor necrosis factor receptor.

Figures

Figure 1

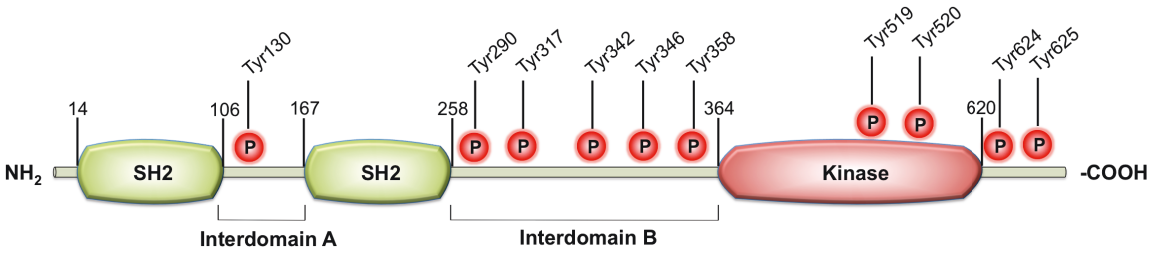
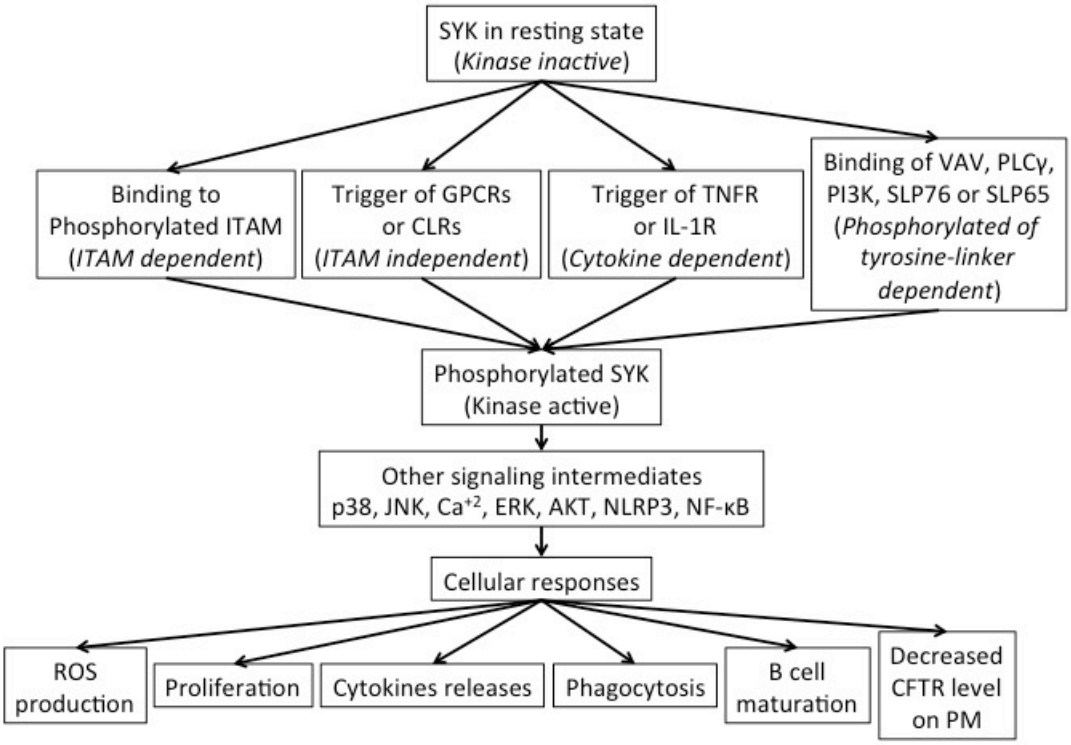


Figure 2


SYK in resting state
(*Kinase inactive*)
Binding to Phosphorylated ITAM
(*ITAM dependent*)
Trigger of GPCRs or CLRs
(*ITAM independent*)
Trigger of TNFR or IL-1R
(*Cytokine dependent*)
Binding of VAV, PLCγ, PI3K, SLP76 or SLP65
(*Phosphorylated of tyrosine-linker dependent*)
Phosphorylated SYK
(Kinase active)
Other signaling intermediates
p38, JNK, Ca^{+2}, ERK, AKT, NLRP3, NF-κB
Cellular responses
ROS production
Proliferation
Cytokines releases
Phagocytosis
B cell maturation
Decreased CFTR level on PM

1.4 Rational, Hypothesis, and Objectives

Through a literature search, no prior research on mutant strains as well as clinical isolates of *P. aeruginosa* from CF patients at different stages of infection has been conducted to explore NLR-mediated innate immune responses to this bacterial infection. This presented us with a unique opportunity for a fundamental molecular innate immunological research. Hence, the objective of this thesis is to appreciate the presence of certain cytosolic sensors: NLRs; which recognize PAMPs and DAMPs. This is to understand what the exact mechanism of *P. aeruginosa* infection to human cells is and how it may be used as a new target therapy. The role of NLRs and inflammasome activations in host immune responses has recently been studied during the last decade and, the current knowledge in this field is limited. Therefore, investigation of the role of NLR-mediated innate immune responses to *P. aeruginosa* will bring new insights into the molecular pathogenesis of bacterial infections. Given that NLRs act in concert with other PRRs in activating innate immune responses to *P. aeruginosa*, the loss of certain virulence factors in the process of host-pathogen interactions will affect the recognition of *P. aeruginosa* by NLRs; this may help bacteria to evade host defenses. Thus, the main goal of this thesis is to clarify the role of NLRs and inflammasome activation in innate immune recognition of *P. aeruginosa*-associated molecular patterns, through NLR-mediated caspase-1 activation and *P. aeruginosa*-induced IL-1β secretion. There are two main objectives in this thesis. First, to determine which *P. aeruginosa* virulence factors are essential for the activation NLR-mediated innate immune responses. Second, to dissect the role of SYK kinase in the regulation of inflammasome activation.

Chapter II: *Pseudomonas aeruginosa* Infection of Human Monocytic Cells Results in Caspase-1 Activation and IL-1β Production

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Abstract

Pseudomonas aeruginosa is the major cause of severe chronic pulmonary disease in cystic fibrosis (CF) patients. NOD-like receptors (NLRs) can recognize a variety of endogenous and exogenous ligands, thereby playing a crucial role in innate immunity. NLR activation initiates inflammasome formation that induces maturation of the pro-inflammatory cytokine interleukin (IL)-1β through activation of caspase-1. We hypothesized that genetic alterations of *P. aeruginosa* affect the innate immune response of human monocytes. THP-1 human monocytic cells were infected with clinical *P. aeruginosa* isolates from CF patients, or with *P. aeruginosa* mutant strains lacking flagella, pili, lipopolysaccharide, or pyocyanin. *P. aeruginosa* isolates from patients with chronic CF lung infection or mutant strains induced lower apoptosis, surface ICAM-1 expression, caspase-1 activation, and proinflammatory cytokine release compared to isolates from CF patients with intermittent *P. aeruginosa* colonization or wild type strains. Our findings suggest that *P. aeruginosa*, which lost certain virulence factors during pulmonary infection, may fail to induce caspase-1 activation and secretion of IL-1β in the

process of host-pathogen interactions. This may reveal novel mechanism of the pathogen adaptation to avoid detection by NLRs.

Keywords: *Pseudomonas aeruginosa*, Cystic Fibrosis, Infection, NOD-like receptors, Caspase-1, Cytokines

2.1 Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterial pathogen that is responsible for chronic lung infection and the main cause of morbidity and mortality in cystic fibrosis (CF) patients [1,2]. Due to great intrinsic and acquired resistance, *P. aeruginosa* has brought a big challenge with current antibiotic therapies [3]. Therefore, understanding of the host immune response against this pathogen has attracted much attention. Lung infection with *P. aeruginosa* is often associated with exaggerated inflammatory responses and characterized by excessive production of various inflammatory cytokines and chemokines including interleukin (IL)-1β [4-7]. As IL-1β is a major inflammatory mediator, its high levels in the bronchoalveolar lavage and sputum of CF patients with *P. aeruginosa* infection are potentially important in the pathogenesis of exacerbations in CF chronic pulmonary disease [8-11].

Maturation and production of IL-1β is tightly controlled by caspase-1 [12-14]. Caspase-1 is regulated by a complex protein structure called inflammasome, which consists of nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR), apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 [15,16]. Once NLR is activated as an intracellular sensor, these components rapidly assemble into inflammasome and recruit pro-caspase-1 for cleavage and activation [17].

Pro-caspase-1, a cytosolic cysteine protease, is constitutively expressed by its producer cells [18] and is activated in various inflammasomes [19]. However, the role of *P. aeruginosa* virulence factors in NLR-mediated activation of caspase-1 in monocytes and macrophages, which are the major source of IL-1β in *P. aeruginosa* infection [4], remains poorly understood.

Given the ability of *P. aeruginosa* to mutate during the process of adaptation to the host, and the important role of NLRs in recognition of *P. aeruginosa*, we hypothesized that the loss of certain virulence factors will affect the recognition of *P. aeruginosa* by NLRs, and this may help bacteria to escape the host defenses. To test this hypothesis, we studied the interactions of well-characterized clinical isolates of *P. aeruginosa* obtained from CF patients during longitudinal observation, or *P. aeruginosa* mutant strains lacking flagella, pili, lipopolysaccharide, or pyocyanin with human THP-1 monocytic/macrophage cells in an *in vitro* model. Previous studies of these clinical isolates showed drastic differences in the expression of virulence factors between bacteria isolated from intermittently colonized *versus* chronically infected CF patients [20] that implies that the interactions between these two groups of bacteria and infected cells can be significantly different. We have examined apoptosis of infected cells, activation of caspase-1, production of proinflammatory cytokines, and adhesion molecule expression as well as used a specific caspase-1 inhibitor. Our findings suggest that loss of virulence factors over the course of chronic infection can result in decreased abilities of recognition of *P. aeruginosa* by NLRs and attenuate NLR-mediated caspase-1 activation; this may confer the persistence of *P. aeruginosa* in CF patients. Our results reveal a novel

mechanism of the pathogen adaptation to avoid detection by NLRs and may provide a better understanding of the host-pathogen interactions during *P. aeruginosa* infection.

2.2 Materials and Methods

2.2.1 Cell culture conditions

Human THP-1 monocytic leukemia cell line (ATCC, Manassas, VA) was stored in liquid nitrogen until thawed for culturing and used at the passage numbers of 6-20. Cells were maintained at 37˚C with 5% CO2 in RPMI-1640 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS) and 1% antibiotic-antimycotic (Invitrogen, Burlington, ON, Canada). Cells were seeded in T-25 cm2 flasks (Corning Incorporated, NY, USA) and passaged every 3-4 days when culture density reached 1 × 106 cells/mL; cells number and viability were determined by using 0.4% Trypan blue solution (Sigma-Aldrich, St. Louis, MO). To induce differentiation, cells were treated with 20 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich) at 37˚C with 5% CO2 for 12 hours, then washed and re-suspended in the same culture medium with FBS and antibiotics. After 48 hours of further incubation, the cells were washed twice and used for experiments with serum- and antibiotic-free medium.

2.2.2 *Pseudomonas aeruginosa* strains and in vitro infectious model

Pseudomonas aeruginosa strain K wild type (PAK WT, provided by Dr. R.J. Irvin, University of Alberta, Edmonton, AB), and the isogenic *P. aeruginosa* mutants PAK NP (pili deficient) and PAK fliC (flagella deficient, provided by Dr. A.S. Prince, Columbia University, New York), PAK rmlC (a lipopolysaccharide mutant with truncated core oligosaccharide) (Provided by Dr. J.S. Lam, University of Guelph, ON), *P. aeruginosa*

PAO1 (provided by Dr. H. Schraft, Lakehead University, Thunder Bay, ON) and its isogenic pyocyanin-deficient mutants Δ*phz*M and Δ*phz*S (provided by Dr. Gee W. Lau, University of Illinois at Urbana-Champaign, Urbana, IL), as well as *P. aeruginosa* clinical isolates from sputum of CF patients were used (Table 1). Among the clinical isolates, 13 were from intermittently colonized individual patients and 14 were from 3 chronically infected patients (the latter obtained during longitudinal observation at the Danish CF Center); all were kindly provided by Dr. N. Høiby (Rigshospitalet, Copenhagen, Denmark). The characteristics of the isolates are described in our previous study [21].

All bacterial strains were maintained in a sterile Luri-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) with 1% agar (LBA). A single colony of *P. aeruginosa* was grown overnight in sterile LB broth at 37˚C on a shaking platform at 150 rpm and diluted by a factor of 20 into fresh LB broth. Cultures were allowed to grow for approximately 1 hr or until mid-log phase when optical density at 600 nm (OD600) reached 0.30. The culture was then centrifuged at 3500 × *g*, for 20 min at 4˚C, and washed twice in sterile phosphate-buffered saline (PBS, pH 7.4). Following the final re-suspension, bacteria were diluted to an OD600 of 0.30 in sterile serum- and antibiotic-free RPMI-1640 medium corresponding approximately to 2 × 108 CFU/mL, as determined by serial dilutions and drop plating on LBA. From this stock, bacteria were added to THP-1 cells at a multiplicity of infection (MOI) of 5 or 10, as was optimized with PAK WT during 1, 2, 6, 12, or 18 hours at 37˚C with 5% CO2 in our previous experiments [22]. For positive control, THP-1 cells were stimulated with either 100 ng/mL *Escherichia coli* LPS

O111:B4 or 5 mM ATP (Sigma-Aldrich) at 37˚C with 5% CO2. All compounds were solubilized in sterile distilled H2O.

2.2.3 Pretreatment with Caspase-1 Inhibitor

THP-1 cells were grown for 24 hours to 1×10^6 cells/mL and a specific caspase-1 inhibitor Ac-YVAD-cmk (N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone, Sigma-Aldrich, Saint-Louis, MO) dissolved in DMSO was added to the medium to achieve a final concentration of 40 μM. The cells were incubated in the presence of Ac-YVAD-cmk for either 2 or 5 hours, then washed twice with PBS and used for experiments. These conditions were developed based on published literature describing Ac-YVAD-cmk pretreatment [23,24] and cellular viability testing using 40 μM of Ac-YVAD-cmk for 2 and 5 hours. No noticeable effect of 40 μM of Ac-YVAD-cmk on cell viability tested during 2, 5, and 18-hour-long incubations was detected (86-96% viable cells).

2.2.4 Analysis of infected cells' viability

THP-1 cells at a concentration of 0.4×10^6 cells/2mL in serum- and antibiotic-free culture medium were stimulated with *P. aeruginosa* at an MOI of 5 for 3 hrs in 12-well plates (Fisher Scientific). Following incubation, the cells were washed with sterile PBS, centrifuged at $500 \times g$ for 5 min, and re-suspended in the same medium. The viability of THP-1 cells was assessed by Trypan Blue Exclusion assay using a Vi-Cell™ XR Viability Analyzer (Beckman Coulter, Mississauga, ON).

2.2.5 Flow cytometry analysis of surface expression of ICAM-1

THP-1 cells were plated at 0.5×10^6 cells/mL in a 24-well plates (Fisher Scientific), and infected with *P. aeruginosa* at an MOI of 5 for 1 hr at 37˚C with 5% CO_2,

then bacteria were killed by adding 100 μg/mL gentamicin (Sigma-Aldrich). Infected cells and dead bacteria were incubated together for a further 17 hrs. Following incubation, cells were washed and resuspended in 100 μl of PBS supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich) containing phycoerythrin (PE)-conjugated monoclonal antibody (mAB) against ICAM-1 (Mouse anti-human CD54, BD Biosciences, Mississauga, Ontario) or mouse IgG1 isotype control at a dilution of 1:50 and incubated for 1 hr at 4˚C. Samples were washed twice with PBS and analyzed by flow cytometry on the FACSCalibur with CELLQUEST PRO software (BD Biosciences, Mississauga, ON, Canada). The data were analyzed using CELLQUEST PRO software and expressed as mean fluorescence intensity (MFI).

2.2.6 Apoptosis Detection

THP-1 cells were plated at 0.5×10^6 cells in 2 mL in 12-well plates (Fisher Scientific), in serum- and antibiotic-free culture medium and incubated in 37˚C, with 5% CO_2 for 24 hrs. Cells were stimulated with *P. aeruginosa* at an MOI of 5 for 3 hrs. Following stimulation, cells were washed with sterile PBS, centrifuged at 500 × *g* for 5 min. Apoptosis was measured by the presence of active caspase-3 and caspase-7, detected by the CaspaTag caspase-3,7 *in situ* assay kit (Chemicon International, Temecula, CA) via flow cytometry according to the manufacturer's protocol. The data were expressed as mean fluorescence intensity (MFI) of active caspase-3,7-expressing cells.

2.2.7 Caspase-1 Detection

For analysis of caspase-1 activation, THP-1 cells were stimulated with *P. aeruginosa* at an MOI of 5 for 3 hrs., then washed with sterile PBS as described above. The presence of active caspase-1 was detected by the FAM-FLICA® *in vitro* Caspase 1

Kit (ImmunoChemistry Technologies, LLC., Bloomington, MN, USA) via flow cytometry according to the manufacturer's protocol. Four quadrants of cell populations were detected: lower left represented unstimulated and unstained cells; lower right, cells in early apoptosis with FLICA stain; upper right, cells in late apoptosis with FLICA and propidium iodide (PI) stains; upper left, necrotic cells with only PI staining. The results were expressed as MFI of active caspase-1-expressing cells.

2.2.8 Western Blotting

For detection of caspase-1 and IL-1β, THP-1 macrophages (2×10^6 cells/mL) were stimulated with PAK WT at an MOI of 5 for 1, 2, or 4 hours, or MOI of 10 and 20 for 2 hours at 37°C, 5% CO_2. Following stimulation, the cells were lysed in 100 µL of ice-cold RIPA lysis buffer, which included PMSF, sodium orthovanadate and protease inhibitor cocktail, and incubated for 30 min at 4°C. Following incubation, the cells were centrifuged at 8,000 × *g* for 10 min and protein lysate was collected. Samples were resolved by 15% SDS-PAGE gel electrophoresis and transferred to a nitrocellulose membrane. Blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, probed with primary antibody, i.e. anti-caspase-1 (D7F10), anti-IL-1β (D3U3E), and β-actin (13E5) antibodies followed by HRP-linked secondary antibody (7074S) (Cell Signaling Technology), and developed using enhanced chemiluminescence. Bands were scanned and images analyzed using ChemiDoc XRS (Bio-Rad). In some cases, the blots were stripped and re-probed with other antibodies.

2.2.9 ELISA assays

To measure the release of caspase-1 and cytokines, THP-1 macrophages were infected with *P. aeruginosa* at an MOI of 10, for 1 h at 37˚C, with 5% CO_2, and then

bacteria were killed by adding 100 μg/mL gentamicin, as described above. Cell culture supernatants were aliquoted and stored at −80°C until analysis. The levels of IL-1β, TNF-α, IL-10, and INF-γ were determined by ELISA from eBioscience (San Diego, CA) and caspase-1 from R&D Systems (Minneapolis, MN). The ELISA kits were used according to the manufacturer's recommendations. The lower detection limits of the assays were 2 pg/mL for IL-1β and IL-10, 4 pg/mL for TNF-α and INF-γ, and 1.24 pg/mL for caspase-1. Samples from three independent experiments were run in triplicate.

2.2.10 Statistics

Data were expressed as mean ± SEM and represented at least 3 independent experiments. All data for intermittent (n = 13) and chronic (n = 14) strains have been pooled together. Statistical significance was determined by one-way analysis of variance (ANOVA) using GraphPad Prism 7.0 (La Jolla, CA, USA). *P* values < 0.05 were considered significant.

2.3 Results

2.3.1 Infection with *Pseudomonas aeruginosa* strains isolated from intermittently colonized CF patients caused reduction in THP-1 cells' viability

To assess the viability of THP-1 monocytic cells following *P. aeruginosa* infection, cells were infected as described in Materials and Methods. The number of live cells was significantly lower in cultures stimulated with 100 ng/mL LPS ($P < 0.01$), or infected with PAK WT (either MOI of 5 or 10) ($P < 0.05$) compared to unstimulated THP-1 cells (figure 1A). However, no difference in viability was noted between cells infected with PAK WT at an MOI of 5 vs MOI of 10 (figure 1A). The viability of THP-1 cells infected with either pili- (PAK NP) or LPS-deficient (PAK rmlC) *P. aeruginosa* did not

significantly differ ($P > 0.05$), and was similar to viability of cells infected with PAK WT (Figure 1B). However, flagella-deficient mutant (PAK fliC) caused a decreased viability of the cells as compared to PAK WT ($P < 0.01$). The viability of cells infected with pyocyanin-deficient PAO1 mutants (either Δ*phz*M or Δ*phz*S) was slightly elevated compared to wild type PAO1 strain but only Δ*phz*S was statistically significant compared to wild type strain ($P < 0.05$). During 3-hr long infection, *P. aeruginosa* clinical isolates from either chronic or intermittent infection caused a significant reduction in cell viability as compared to PAK WT ($P < 0.0001$). However, the clinical isolates from intermittently colonized CF patients were capable to further reduce the viability of cells as compared to the isolates from chronically colonized CF patients ($P < 0.01$). These data suggest that the ability of *P. aeruginosa* to reduce the viability of monocytic cells depends on the presence of major cell-associated virulence factors.

2.3.2 Apoptosis induced by *P. aeruginosa* is significantly higher when THP-1 cells are infected with strains isolated from intermittently infected CF patients compared to chronically infected ones

As we have previously found that apoptosis of *P. aeruginosa* infected human lung epithelial cells depends on the presence of specific virulence factors, such as pili, LPS, or flagella, we extended the analysis to human monocytic cells [21]. We have detected cells committed to apoptosis using a fluorescence-labelled peptide, which specifically binds to active caspase-3,7 [25]. Three-hour-long infection of THP-1 monocytic cells with PAK WT ($P < 0.05$) induced higher expression of active caspase-3,7 in comparison to the effect of LPS suggesting that in addition to LPS, other bacterial components may contribute to cellular apoptosis (figure 2). Stimulation with *P. aeruginosa* strains, i.e.

PAK WT, pili-deficient (PAK NP), flagella-deficient (PAK fliC), LPS-deficient (PAK rmlC), PAO1, PAO1 pyocyanin-deficient mutants (Δ*phz*M and Δ*phz*S), or strains isolated from intermittently colonized and chronic infected CF patients caused greater intracellular expression of active caspase-3,7 compared to unstimulated cells ($P < 0.001$, figure 2), indicating that infected cells were undergoing apoptosis. However, the expression of active caspase-3,7 was higher in the case of infection with strains isolated from intermittently colonized CF patients compared to infection with 'chronic' strains ($P < 0.01$, Figure 2).

2.3.3 *P. aeruginosa* infection decreases pro-caspase-1 and increases pro-IL-1β expressions in THP-1 macrophages

To examine if inflammasome activation occurs in THP-1 macrophages infected with *P. aeruginosa*, we studied first the expression of pro-caspase-1 and pro-IL-1β using Western blot (Figure 3A – D). Previous studies showed that both LPS and ATP induced caspase-1 activation in THP-1 monocytic cell line [4,26]. Stimulation of THP-1 macrophages with LPS ($P < 0.001$) or ATP in the presence of LPS ($P < 0.0001$) induced significant up-regulation of both pro-caspase-1 and pro-IL-1β compared to unstimulated cells (Figure 3A and C). Stimulation with PAK WT at MOI of 5 during 1, 2, or 4 hours led to a decreased expression of pro-caspase-1 compared with LPS ($P < 0.001$) or ATP in the presence of LPS ($P < 0.0001$) (Figure 3A). However, expression of pro-caspase-1 during 2 hours stimulation with PAK WT at MOI 5 showed the only statistically significant difference in comparison to un-stimulated cells. Therefore, we extended our analysis using higher MOIs (i.e., 10 and 20) during 2-hour stimulation. While infection at MOI of 10 induced similar expression of pro-caspase-1 as in unstimulated cells, the

difference was statistically significant at MOI of 20 (Figure 3B). In contrast to pro-caspase-1 expression, infection with PAK WT at MOI of 5 for 1 hour ($P < 0.05$), 2 ($P < 0.001$), or 4 hours ($P < 0.0001$), or at MOI of 10 and 20 for 2 hours ($P < 0.0001$) led to significantly higher expression of pro-IL-1β compared to LPS stimulation (Figure 3C-D).

2.3.4 The presence of major cell-associated and secreted virulence factors of *P. aeruginosa* results in increased abilities of bacteria to activate caspase-1

To confirm the activation of caspase-1 by *P. aeruginosa* in our model, we used a fluorescent inhibitor probe to label active caspase-1 together with propidium iodide (PI) to distinguish between viable and non-viable cells, and two-color flow cytometry for analysis.

Representative flow cytometry dot plots are illustrated in Figure 4A-H. For analysis, 3 control cell populations were used, i.e. un-stimulated and un-stained THP-1 monocytic cells (Figure 4A), un-stimulated cells with PI stain to distinguish between living and dead cells (Figure 4B), and un-stimulated cells with bicolor staining, i.e. PI and FLICA (Figure 4C). FLICA was used for active caspase-1 staining. To further confirm PI and FLICA staining, THP-1 monocytic cells were treated with 90% ETOH to discriminate between viable and non-viable cells via PI staining (Figure 4D), or with 5 mM ATP to activate caspase-1 via FLICA staining (Figure 4E). Two-color staining with PI and FLICA was used for cells treated with both 90% ETOH and ATP (Figure 4F), 100 ng/mL LPS (Figure 4G), or infected with PAK WT (Figure 4H).

Three hour-long infection of THP-1 cells with PAK WT resulted in a significant increase in the expression of active caspase-1 as compared to treatment with LPS (Figure 4I). While PAK WT (607.1 ± 42.96) and PAO1 (650.8 ± 78.11) induced similar level of

active caspase-1 expression, *P. aeruginosa* mutant strains, i.e. pili-deficient (PAK NP), flagella-deficient (PAK fliC), and LPS-deficient (PAK rmlC), or PAO1 pyocyanin-deficient mutants (Δ*phz*M and Δ*phz*S) induced lower levels of active caspase-1 compared to stimulation with their isogenic wild type strains (PAK WT or PAO1, respectively) (Figure 4I). Clinical isolates obtained from intermittently colonized CF patients induced significantly lower active caspase-1 expression ($P < 0.05$) compared to PAK WT (Figure 4I). The isolates obtained from chronically infected patients showed further reduced abilities to activate caspase-1 similar to the effect of mutant strains lacking the major virulence factors (Figure 4I).

To further confirm the activation of caspase-1, we stimulated THP-1 macrophages with *P. aeruginosa* for 18 hours (1 hour infection, followed by 17 hours of incubation with bacteria in the presence of gentamicin). ELISA data showed that presence of active caspase-1 in culture supernatants of stimulated THP-1 macrophages (Figure 5A) were consistent with detection of active caspase-1 in THP-1 monocytic cells using flow cytometry (Figure 4I). Despite a different degree of caspase-1 activation induced by *P. aeruginosa* strains, the absence of specific virulence factor(s) was associated with decreased caspase-1 activation (Figure 5A). Moreover, pretreatment of cells with a specific caspase-1 inhibitor for 2 or 5 hours prior to *P. aeruginosa* infection greatly decreased the release of active caspase-1 (Figure 5B).

2.3.5 Lack of specific cell-associated or secreted virulence factors of *P. aeruginosa* results in decreased inflammatory responses of THP-1 cells

To study the release of cytokines and expression of ICAM-1, we stimulated THP-1 macrophages with *P. aeruginosa* as described in Materials and Methods. Our preliminary

experiments indicated that THP-1 monocytic cells had low capacities of cytokine production (data not shown). Stimulation with all *P. aeruginosa* strains induced a significant increase in surface ICAM-1 expression by THP-1 macrophages (Figure 6). In comparison to PAK WT, stimulation with the isogenic mutants PAK NP ($P < 0.001$), PAK rmlC ($P < 0.01$), and PAK fliC ($P < 0.01$) resulted in lower expression of ICAM-1 (Figure 6) suggesting that the lack of specific virulence factor(s) may account for this effect. We observed similar phenomenon when the infection with the isogenic *P. aeruginosa* PAO1 mutants Δ*phz*M ($P < 0.01$), and Δ*phz*S ($P < 0.001$) was compared to the wild type PAO1 strain (Figure 6). The expression of ICAM-1 in infected cells was higher in the case of infection with strains isolated from intermittently colonized CF patients compared to infection with 'chronic' strains ($P < 0.05$, Figure 6).

Similar to the surface ICAM-1 expression, we observed a higher release of IL-1β and TNF-α by cells stimulated with PAK WT, PAO1, and isolates from intermittently colonized CF patients compared to infection with isogenic *P. aeruginosa* mutants or 'chronic' strains (Figure 7A,B). These data corroborate our findings that the lack of specific virulence factors resulted in a significant attenuation of caspase-1 activation. IL-10 release was significantly increased in cells infected with PAK WT, PAO1 mutant (Δ*phz*M), and both types of clinical isolates as compared to the un-stimulated cells (figure 7C). IFN-γ was statistically significantly induced by PAO1 and PAO1 mutant (Δ*phz*M) in comparison to un-stimulated cells (figure 7D). Isolates from chronically infected CF patients induced a significant increase of IFN-γ release in comparison to un-stimulated cells ($P < 0.001$) or cells infected with isolates from intermittently colonized CF patients ($P < 0.0001$) (figure 7D).

In the next experiments, we tested the effect of a specific caspase-1 inhibitor Ac-YVAD-cmk, which is reportedly effective in concentration of 40 μM in THP-1 cell culture condition [24]. Cell-surface expression of ICAM-1 in THP-1 macrophages pretreated with the inhibitor and stimulated with PAK WT at an MOI of 5, or LPS was similar to stimulated cells without pretreatment (data not shown). However, caspase-1 inhibitor pretreatment of THP-1 macrophages stimulated with LPS, LPS + ATP, PAK WT or its isogenic mutants resulted in a significant attenuation of IL-1β release, with no significant difference between 2 and 5 hours of pretreatment (Figure 8A). In contrast, under the same conditions, no effect of caspase-1 inhibitor on the release of TNF-α was observed (Figure 8B).

2.4 Discussion

Early *P. aeruginosa* infection in an immunocompetent host involves a variety of functional cell types and mediators that recognize the pathogen and initiate innate immunity, eventually clearing the pathogen from the airways [27-29]. Individuals with mutation in *CFTR* have dehydrated and thickened airway surface liquid that hinders mucociliary clearance. The natural history of progression of lung disease in CF individuals shows susceptibility to pulmonary *P. aeruginosa* infection; the disease starts with an initial acute infection and vigorous inflammatory response, followed by chronic respiratory infection (CRI) [30,31]. *P. aeruginosa* isolates from CF patients with CRI undergo remarkable phenotypic and genotypic alterations, i.e. lose of a number of virulence factors, change the phenotype, form biofilm, and acquire resistance to many antibiotics. Several studies have suggested those alterations may confer survival advantages to *P. aeruginosa* in CRI and benefit persistent bacterial colonization

[20,30,32-40]. These observations suggest that there is a complex interplay between *P. aeruginosa* virulence factors and host defense mechanisms in CRI caused by *P. aeruginosa*.

In this study, we hypothesized that the genetic diversification acquired by *P. aeruginosa* during the course of CRI may alter NLR-mediated caspase-1 activation in innate immune cells resulting in decreased host defenses. We first studied the viability of THP-1 monocytic cells infected with *P. aeruginosa* and established that the strains isolated from intermittently colonized CF patients caused a decreased THP-1 viability as compared to isolates from chronically infected CF patients. This could be related to their different abilities to become internalized as was demonstrated by our previous studies in a model of infected lung epithelial cells (Hawdon et al., 2009). Recent studies found that *P. aeruginosa* are cytotoxic to THP-1 monocytes [24] and THP-1 macrophages [41]. Engulfment and interactions of *P. aeruginosa* with phagocytic cells are mediated by a number of ligands expressed on the pathogen and numerous host receptors, for example asialo GM1 and TLRs [42-50]. Our data showed that pili, lipopolysaccharide, or blue pigment pyocyanin were not essential for decreasing cell viability because the mutant strains lacking any of these virulence factors caused similar decrease in cell viability as their isogenic wild type strains. However, infection with a flagella-deficient mutant resulted in decreased cell viability, which may be related to its reduced ability to activate the transcription factor NF-κB. Bacterial flagellin recognized by TLR5 and NLRC4 is known to activate the NF-κB pathway [51,52]. Indeed, our data showed that a flagella-deficient strain induced significantly lower expression of ICAM-1 (Figure 6), release of proinflammatory cytokines IL-1β (Figure 7A) and TNF-α (Figure 7B), and caspase-1

(Figures 4I & 5A,B) compared to the wild type strain. While NF-κB regulates pro-inflammatory responses [53], it also regulates cell survival [54], and this may explain why cells infected with flagella-deficient mutant show a decreased viability compared to the wild type *P. aeruginosa*

Apoptosis was found to be necessary for protective host response against *P. aeruginosa* infection *in vivo* [55]. *P. aeruginosa* can cause apoptosis of host cells via both death-receptor associated (extrinsic) and mitochondrial (intrinsic) pathways [55,56]. Using the detection of active effector caspase-3,7 involved in both pathways, we found that *P. aeruginosa* strains causing chronic pulmonary infection induced less apoptosis, compared with strains causing intermittent colonization. These data suggest that diminished apoptosis of infected cells can contribute to the persistence of *P. aeruginosa* infection. These findings are consistent with our previous observations on lung epithelial cells [21]. However, in present study, *P. aeruginosa* mutants induced similar activation of caspase-3,7 as their wild type strains suggesting the role of some virulence factors apart from pili, flagella, LPS and pyocyanin, in inducing apoptosis of infected monocytic cells. While the role of pili, flagella, LPS, and the effectors of the T3SS in apoptosis caused by *P. aeruginosa* has been previously demonstrated [57-59], some studies suggest that *P. aeruginosa* may induce different forms of cell death by necrosis (caspase-1 and -3 independent), oncosis (caspase-3 independent) or pyroptosis (caspase-1 dependent) [24,60]. These discrepancies may depend on the experimental model and need further investigation.

ICAM-1, an adhesion molecule critical in the recruitment of inflammatory cells to the infected tissue, is constitutively expressed at low levels on macrophages [61], but not

on circulating monocytes. However, its expression can significantly rise in the presence of proinflammatory cytokines such as TNF-α, or bacterial virulence factors [62-65]. We found that *P. aeruginosa* from early stages of CF pulmonary infection and wild type strains induced higher surface expression of ICAM-1, compared to the strains isolated from chronically infected patients and mutant strains. As *P. aeruginosa* wild type strains and clinical isolates from intermittently colonized CF patients tend to induce more proinflammatory cytokines compared with mutant strains and isolates from chronic lung infection, we found consistency in ICAM-1 (Figure 6) and proinflammatory cytokines IL-1β and TNF-α expression (Figure 7A,B).

Once established, *P. aeruginosa* infection leads to an escalation of inflammation, severe tissue damage, and deterioration of lung function [2,27,66]. IL-1β is a potent inflammatory cytokine and its level is increased in bronchoalveolar lavage and sputum of immunocompromised individuals and CF patients with *P. aeruginosa* infection [67-69]. Previous studies have demonstrated that monocytes and macrophages are the primary source of IL-1β production in *P. aeruginosa* infection [4,12]. It was documented that the inflammatory response to *P. aeruginosa* is initiated following the recognition of pathogen-associated molecules by TLRs on the cell surface and NLRs within the cytosol. However, specific mechanisms of NLR-mediated caspase-1 activation in response to microbial stimuli remain poorly understood [70,71]. Following NLR activation, the inflammasome assembles and mediates the activation of caspase-1, which leads to proteolytic processing of immature pro-IL-1β to its biological active form [16,19,72,73]. Production of IL-1β requires two signals: initial one by the activation of TLR, which leads to pro-IL-1β production. The second signal is by the activation of NLR, which

initiates inflammasome formation and caspase-1 activation for release of IL-1β [74]. Our Western blotting results showed that pro-caspase-1 is expressed in unstimulated THP-1 macrophages. In our PAK WT infection model, the expression of pro-IL-1β, but not of pro-caspase-1, was time- and dose-dependent. Stimulation with PAK WT led to a decreased expression of pro-caspase-1 compared with LPS or ATP + LPS, which may be attributed to proteolytic cleavage of pro-caspase-1 into enzymatically active caspase-1. Studies showed that caspase-1 has high substrates specificity at low concentration [75], as well as its overexpression induced autophagy [12].

The activation of caspase-1 following *P. aeruginosa* infection in human monocytic and macrophage cells has recently been documented [4,12], but the role of specific virulence factors or source of clinical *P. aeruginosa* isolates have not been fully elucidated. We found that *P. aeruginosa* infection resulted in the activation of caspase-1 in THP-1 monocytic cells and production of active caspase-1 in THP-1 macrophages; in both models, it was dramatically reduced with mutant strains or clinical isolates from chronically infected CF patients. Different types of NLRs can be activated in innate immunocytes during bacterial infection [76]. It was recently demonstrated that NLRP3, ASC, and caspase-1 were activated in human macrophages following *P. aeruginosa* infection, and their overexpression triggered autophagy, which impaired phagocyte killing [12]. Most importantly, *P. aeruginosa*-induced IL-1β secretion was significantly down-regulated in NLRP3-deficient human macrophages [12]. In contrast, previous studies showed that in mouse macrophages, NLRP3 was not crucial for *P. aeruginosa*-induced IL-1β secretion, in contrast to NLRC4 [74]. Recent studies demonstrated that *P. aeruginosa*-induced IL-1β secretion was unaffected in AIM2-deficient mouse

macrophages; however, AIM2 gene expression and degradation of AIM2 protein were induced by *P. aeruginosa* infection [7]. Further studies are required to understand molecular mechanisms involved in inflammasome activation by *P. aeruginosa*

Caspase-1 inhibitors are commonly used to investigate inflammasome activation [12,24,72,77-79]. In our experiments, pretreatment of THP-1 macrophages with a specific caspase-1 inhibitor significantly inhibited both caspase-1 and IL-1β secretion. While TNF-α is induced by *P. aeruginosa* infection its production is largely independent on NLR-mediated activation pathways [80]. Indeed, in our model, caspase-1 inhibitor did not cause significant decrease of TNF-α release induced by any stimulation (Figure 8B), although some studies showed that active caspase-1 can activate NF-κB, which is responsible for TNF-α gene expression [23,81]. Taken together, our results support that *P. aeruginosa* infection of THP-1 macrophages activates the NLR(s). While the role of other PRRs, such as TLRs, in pathogen immunity has been relatively well studied, the NLR contribution to host-pathogen interactions is less defined. It is worth mentioning that our findings corroborate recent data by others who used a caspase-1 inhibitor [24] or small interfering RNAs to treat THP-1 cells [12].

We found that *P. aeruginosa* infected THP-1 macrophages produced low amounts of IL-10 and IFN-γ. Signals, which regulate production of IL-10, are initiated by the engagement of several PRRs [82], while production of IFN-γ by THP-1 macrophages requires IL-12 in combination of IL-18 as additional signals [83].

In conclusion, our study of cellular responses to clinical *P. aeruginosa* isolates from CF patients obtained at different stages of lung infection along with mutant strains helps to understand the role of NLR activation in innate immune responses to this pathogen. In

particular, we found that *P. aeruginosa* strains isolated from the chronic stage of CF pulmonary infection have decreased abilities to induce caspase-1 activation and IL-1β secretion. As these bacteria were isolated from chronically infected patients, the loss of specific virulence factors during CRI likely enables or reflects *P. aeruginosa* ability to persist in the lung environment. Ability to avoid detection by NLRs may represent an important mechanism of immune evasion. Because clinical isolates during the chronic stage still continuously trigger inflammatory responses this can account for progressive deterioration of lung function in CF [84-91].

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Table

Table 1: Strains of *Pseudomonas aeruginosa* used in this study

Strain Isolate	Sources/References
Wild-type PAK (PAK WT)	R.J. Irvin/[89]
Pili-deficient PAK (PAK NP)	A.S. Prince/[90]
LPS-deficient PAK (PAK rmlC)	J.S. Lam/[91]
Flagella-deficient PAK (PAK fliC)	A.S. Prince/[43]
Wild-type PAO1	H. Schraft/[92]
Pyocyanin-deficient PAO1 (Δ*phz*M)	G.W. Lau/[93]
Pyocyanin-deficient PAO1 (Δ*phz*S)	G.W. Lau/[93]
Isolates from intermittently colonized CF patients	Danish CF Centre/[21]
Isolates from chronically infected CF patients	Danish CF Centre/[21]

Figure Legend

Figure 1 Viability of THP-1 cells infected with *Pseudomonas aeruginosa*.

A. THP-1 monocytic cells were stimulated with LPS or *P. aeruginosa* strain K (PAK WT) at an MOI of 5 or 10 for 3 h, and then viable cells were counted. B. THP-1 monocytic cells were infected with mutant or wild type strains, or individual clinical isolates (27 isolates in total) at an MOI of 5. All data for intermittent (n = 13) and chronic (n = 14) strains have been pooled together. Un-stimulated THP-1 monocytic cells served as a negative control. For figure 1A, ##, $P < 0.01$ compared between LPS and control; #, $P < 0.05$ compared between PAK WT at an MOI of 5 or 10 to control. For figure 1B, #, $P < 0.05$ compared between PAK WT, PAK NP, or PAK rmlC to control; ##, $P < 0.01$ compared between ΔphzM to control; ###, $P < 0.001$ compared between PAO1 to control; **, $P < 0.01$ compared between PAK fliC to PAK WT; +, $P < 0.05$ compared between ΔphzS and PAO1; •••, $P < 0.001$ compared between chronic and intermittent strains.

Figure 2 Apoptosis of THP-1 cells caused by *Pseudomonas aeruginosa* depends on the stage of pulmonary infection in CF patients.

THP-1 monocytic cells were stimulated with *P. aeruginosa* clinical isolates from CF patients, PAK WT and PAO 1, or their isogenic mutant strains. Expression of active caspase-3,7 as indication of apoptosis was detected using flow cytometry. The results were expressed as mean fluorescence intensity (MFI). Un-stimulated THP-1 monocytic cells served as a negative control. ###, $P < 0.001$ compared between LPS, PAK WT, PAK NP, PAK rmlC, PAK fliC, PAO1, Δ*phz*M, or Δ*phz*S and control; ••, $P < 0.01$

clinical isolates from chronically infected CF patients compared to intermittently colonized.

Figure 3 The effect of *P. aeruginosa* infection on the expression of procaspase-1 and pro-IL-1β.

THP-1 macrophages were stimulated with *P. aeruginosa* strain K (PAK WT) either at an MOI of 5 for 1, 2, or 4 hours (A and C), or for 2 hrs at MOI of 10 and 20 (B and D). Following stimulation, pro-caspase-1 (48 kDa) and pro-IL-1β (31 kDa) were detected in cellular lysates by Western blot. Results are expressed as ratios of 48 kDa and 31 kDa band intensity to β-actin. Control: un-stimulated THP-1 macrophages. Results represent the mean ± SEM of 2 independent experiments; #, $P < 0.05$ PAK WT (MOI 20) compared to control; ###, $P < 0.001$ LPS or PAK WT (2 hrs) compared to control ; ####, $P < 0.0001$ ATP in the presence of LPS compared to; º, $P < 0.05$ PAK WT (1 hr) compared to LPS; ººº, $P < 0.001$ ATP in the presence of LPS or PAK WT (2 hrs) compared to LPS; ºººº, $P < 0.0001$ PAK WT (4 hr), PAK WT (MOI 10), or PAK WT (MOI 20) compared to LPS; n.s, not statistically significant.

Figure 4 Effect of *Pseudomonas aeruginosa* infection on the expression of active caspase-1 in THP-1 cells.

THP-1 monocytic cells were stimulated with *P. aeruginosa* clinical isolates from CF patients, PAK WT, PAO 1, or their isogenic mutant strains. Expression of active caspase-1 was detected using flow cytometry as described in Materials and Methods. In figure 4A-H, original representative flow cytometric dot plots are shown; A, un-stimulated and un-stained THP-1 monocytic cells; B, un-stimulated cells with PI stain; C, un-stimulated cells with PI and FLICA stains; D, 90% ETOH-treated cells with PI stain; E, ATP-treated

cells with FLICA stain; F, cells treated with 90% ETOH in the presence of 5 mM ATP; G, cells stimulated with LPS, H,, cells infected with PAK WT stained with PI and FLICA. In figure 4I, ##, $P < 0.01$ LPS compared to control; ####, $P < 0.0001$ PAK WT, PAO1, or clinical isolates from intermittently colonized compared to control; *, $P < 0.05$ PAK NP or PAK rmlC compared to PAK WT; **, $P < 0.01$ PAK fliC compared to PAK WT; +, $P < 0.05$ Δ*phz*M or Δ*phz*S compared to PAO1; •••, $P < 0.001$ clinical isolates from chronically infected CF patients compared to intermittently colonized. Results of three independent experiments are shown. FLICA stain is on FL1; PI stain is on FL2; Control: unstimulated cells.

Figure 5 Effect of *P. aeruginosa* infection on the release of active caspase-1 by THP-1 macrophages.

THP-1 macrophages were infected with *P. aeruginosa* at an MOI of 10 for 1 h followed by 17 h incubation with gentamicin. A, caspase-1 levels in cell supernatants were examined by ELISA, and B, following pretreatment with 40 μM of the specific caspase-1 inhibitor Ac-YVAD-cmk for 2 or 5 hours. In figure 5A, Control: un-stimulated THP-1 macrophages. ###, $P < 0.001$ clinical isolates from intermittently colonized compared to control; ####, $P < 0.0001$ PAK WT or PAO1 compared to control; º, $P < 0.05$ ATP in the presence of LPS compared to LPS; *, $P < 0.05$ PAK NP, PAK rmlC, or PAK fliC compared to PAK WT; +, $P < 0.05$ Δ*phz*S compared to PAO1; ++, $P < 0.01$ Δ*phz*M compared to PAO1; ••, $P < 0.01$ clinical isolates from chronically infected CF patients compared to intermittently colonized. In figure 5B, stimulated THP-1 macrophages without caspase-1 inhibitor pretreatment served as a control. Release of caspase-1 by

cells pretreated with the inhibitor prior to stimulation in comparison to control, $P < 0.001$; ΔΔΔΔ, $P < 0.0001$. n.s, not statistically significant.

Figure 6 Surface expression of the adhesion molecule ICAM-1 on THP-1 macrophages following *Pseudomonas aeruginosa* infection.

Analysis of cell surface expression of ICAM-1 was performed by flow cytometry following infection with *P. aeruginosa* at an MOI of 5. Un-stimulated THP-1 macrophages served as a negative control. ####, $P < 0.0001$ LPS, PAK WT, PAO1, or clinical isolates from intermittently colonized compared control; **, $P < 0.01$ PAK rmlC or PAK fliC compared to PAK WT; ***, $P < 0.001$ PAK NP compared to PAK WT; ++, $P < 0.01$ Δ*phz*M compared to PAO1; +++, $P < 0.001$ Δ*phz*S compared to PAO1; •, $P < 0.05$ clinical isolates from chronically infected CF patients compared to intermittently colonized.

Figure 7 Cytokine release from THP-1 macrophages stimulated with *Pseudomonas aeruginosa* for 18 h.

THP-1 macrophages were infected with *P. aeruginosa* clinical isolates from CF patients, PAK WT and PAO 1, or their mutant strains at an MOI of 10. Un-stimulated THP-1 macrophages served as a negative control. Concentrations of IL-1β (A), TNF-α (B), IL-10 (C), and IFN-γ (D) in cell supernatants were measured by ELISA. Data represent mean cytokine concentration ± SEM (n=3). #, $P < 0.05$ PAK WT, PAO1, or Δ*phz*M compared to un-stimulated THP-1 macrophages; ##, $P < 0.01$ LPS compared to control; ###, $P < 0.001$ ATP in the presence of LPS, or clinical isolates from chronically infected CF patients compared to control; ####, $P < 0.0001$ LPS, PAK WT, PAO1, clinical isolates from intermittently colonized or chronically infected CF patients compared to

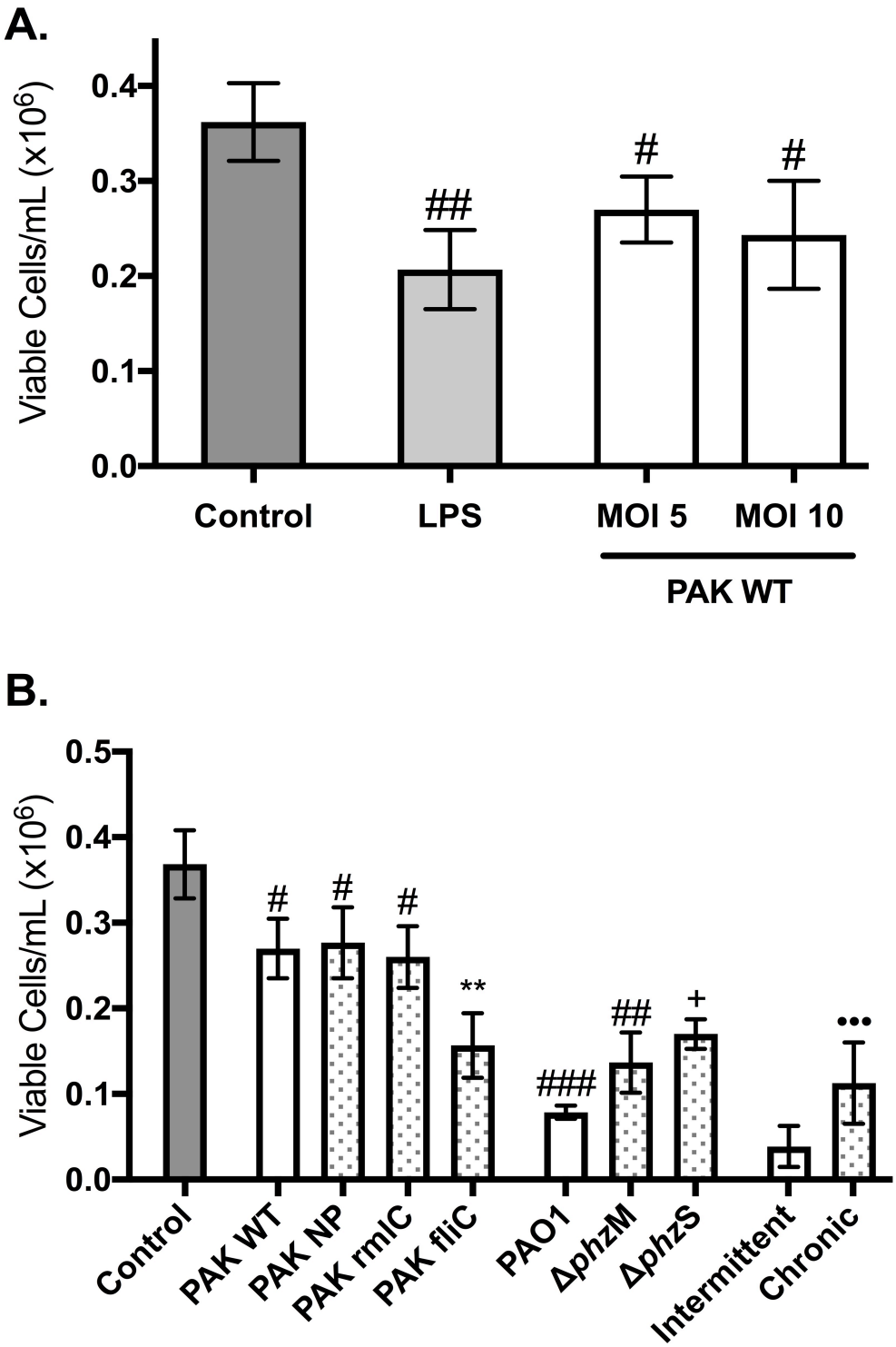
control; **, $P < 0.01$ PAK NP, PAK rmlC, or PAK fliC compared to PAK WT; ***, $P < 0.001$ PAK rmlC compared to PAK WT; ****, $P < 0.0001$ PAK NP or PAK fliC compared to PAK WT; ++++, $P < 0.0001$ Δ*phz*M or Δ*phz*S compared to PAO1; ••••, $P < 0.01$ clinical isolates from chronically infected CF patients compared to intermittently colonized.

Figure 8 Effect of caspase-1 inhibitor on cytokine production by THP-1 macrophages infected with *P. aeruginosa*.

THP-1 macrophages were pre-incubated with 40 μM of the specific caspase-1 inhibitor Ac-YVAD-cmk for either 2 or 5 hours and then infected with *P. aeruginosa* PAK WT or its isogenic mutant strains at an MOI of 10. Stimulated THP-1 macrophages without pretreatment served as a control. Concentrations of IL-1β (A) and TNF-α (B) were measured by ELISA. Data represent mean cytokine concentration ± SEM (n=3). Release of cytokines by cells pretreated with the inhibitor prior to stimulation in comparison to control, Δ, $P < 0.05$; ΔΔ, $P < 0.01$; ΔΔΔ, $P < 0.001$; ΔΔΔΔ, $P < 0.0001$. n.s, not statistically significant.

Figures

Figure 1



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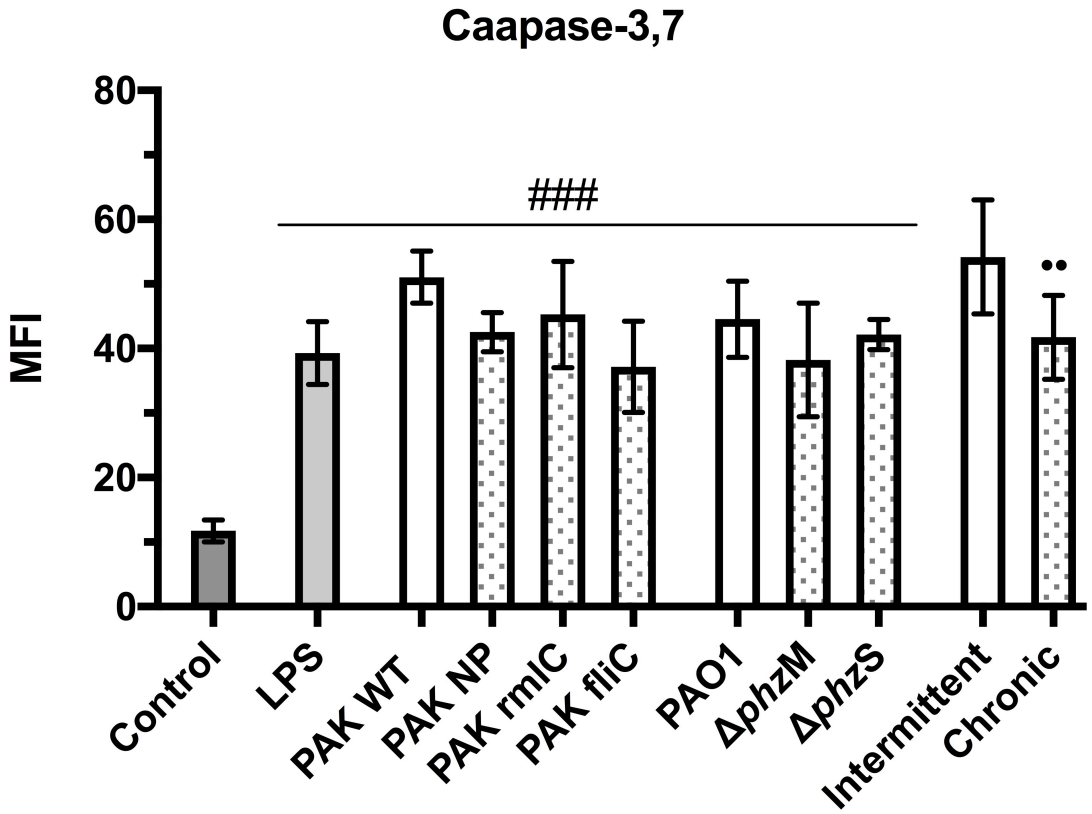
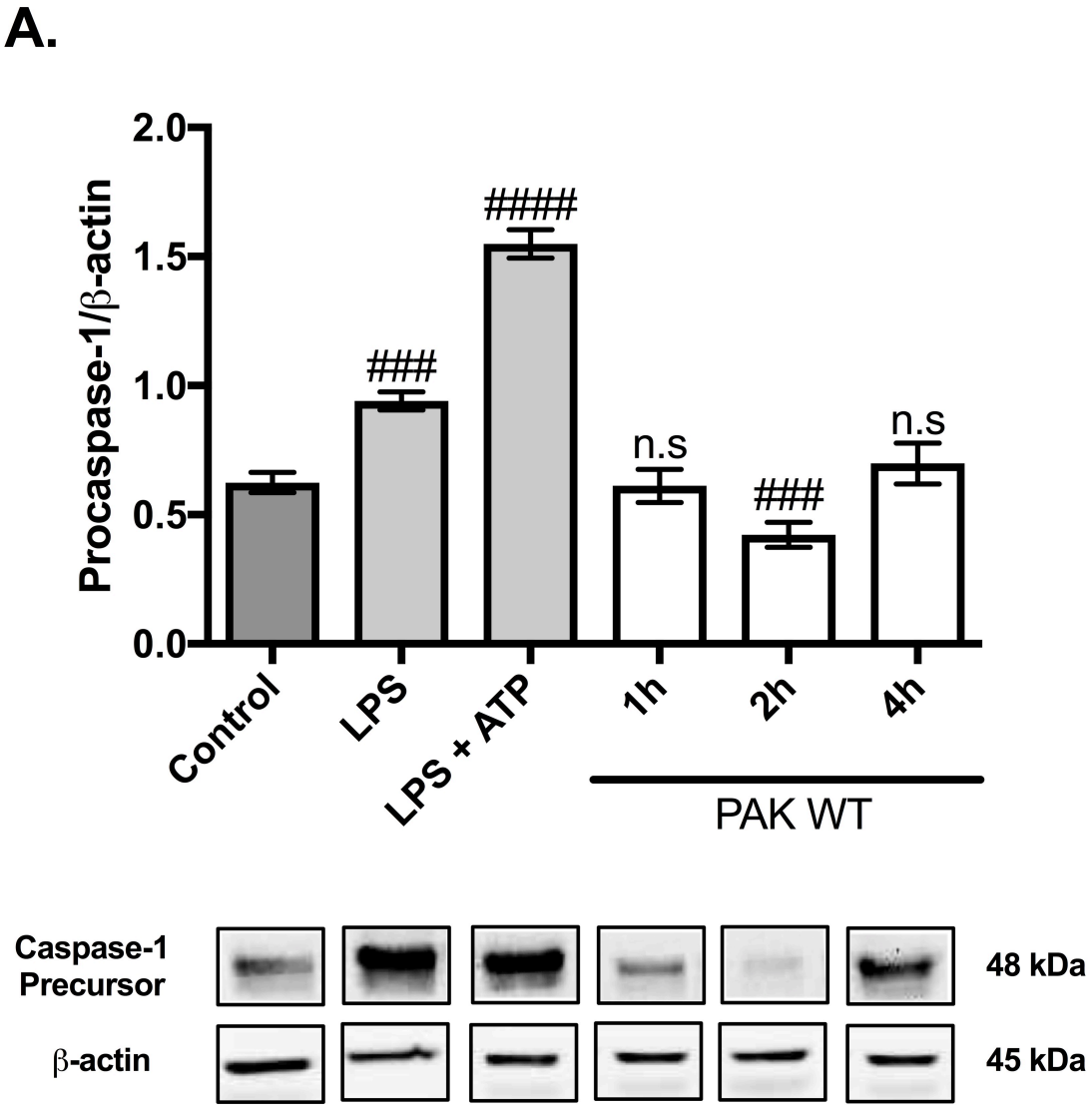
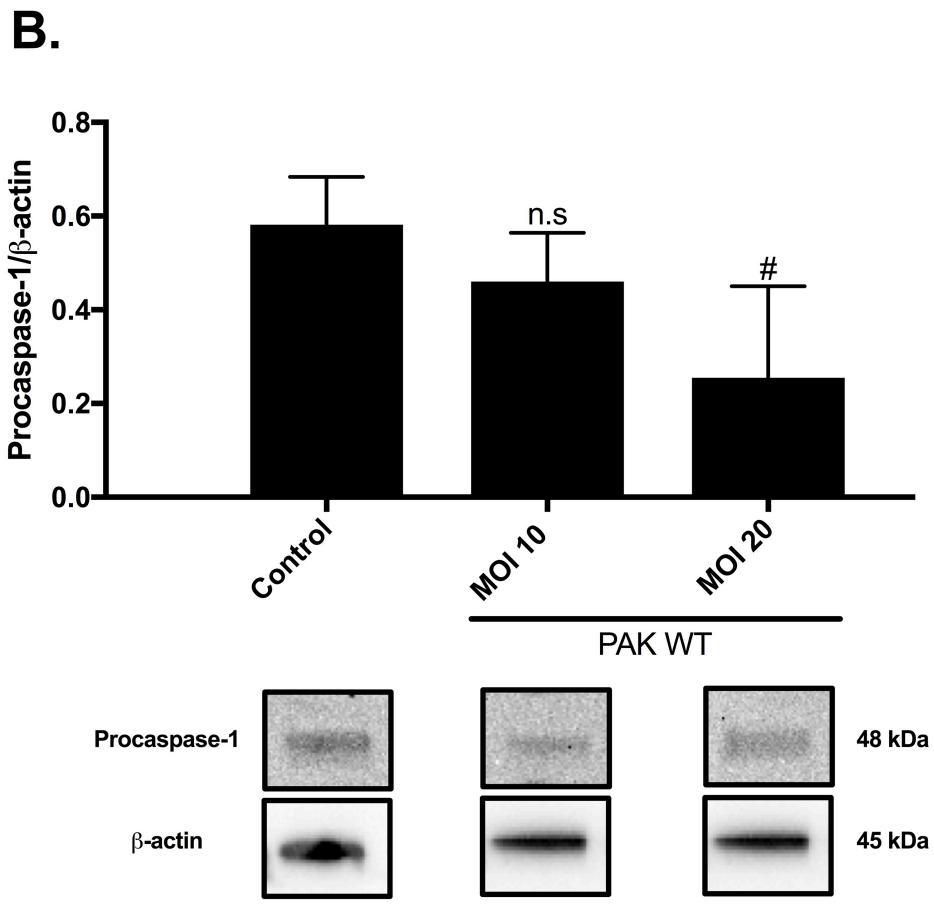

Caapase-3,7
MFI
80
60
40
20
0
###
••
Control
LPS
PAK WT
PAK NP
PAK rmlC
PAK fliC
PAO1
ΔphzM
ΔphzS
Intermittent
Chronic

Figure 3


A.
Procaspase-1/β-actin
2.0
1.5
1.0
0.5
0.0
###
####
n.s
###
n.s
Control
LPS
LPS + ATP
1h
2h
4h
PAK WT
Caspase-1 Precursor
β-actin
48 kDa
45 kDa

Cotinue Figure 3



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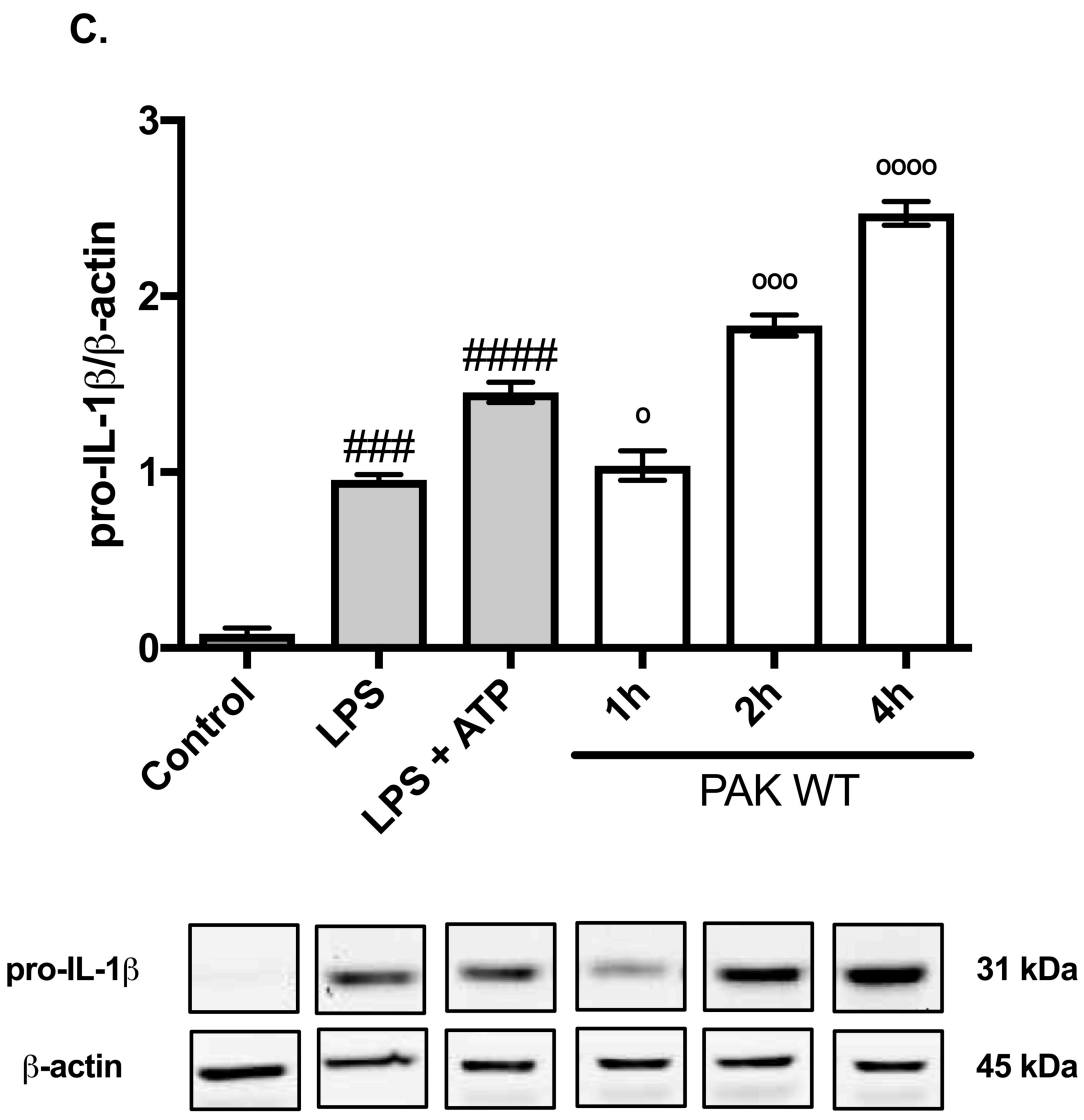

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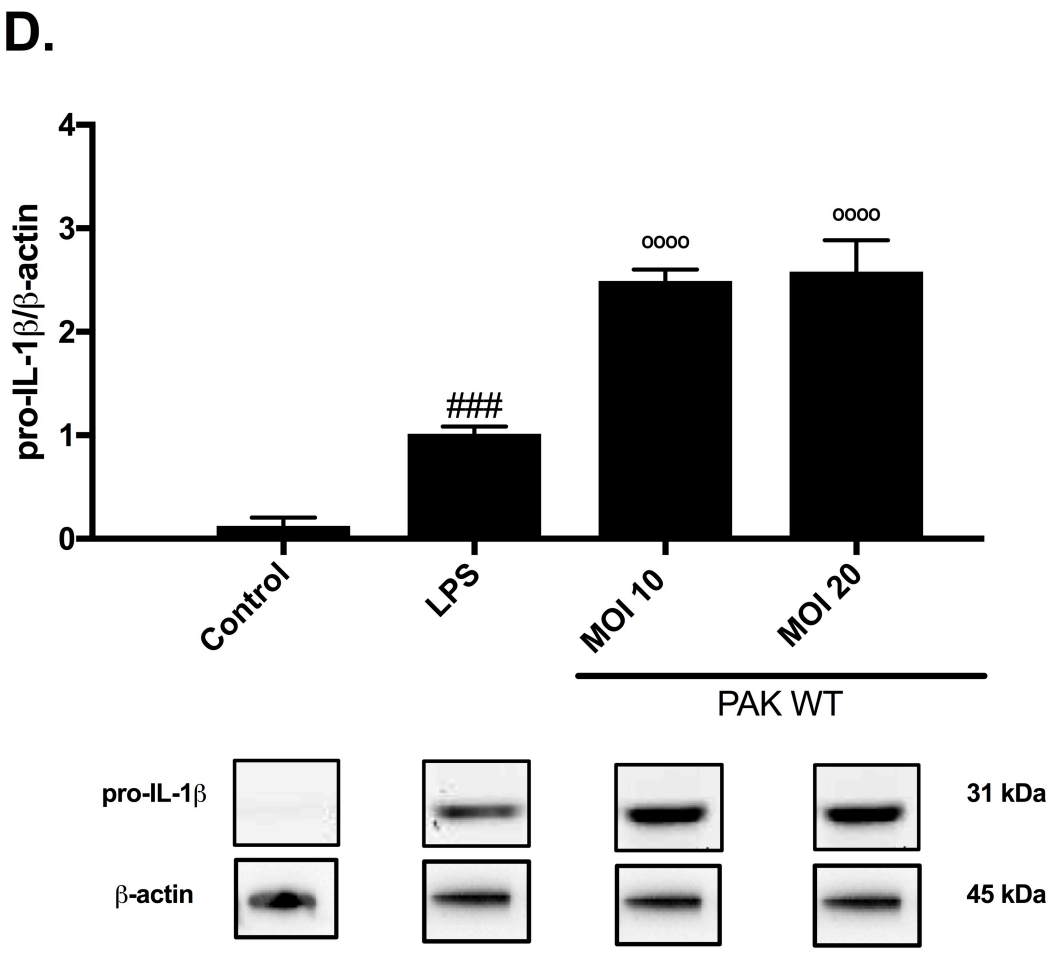
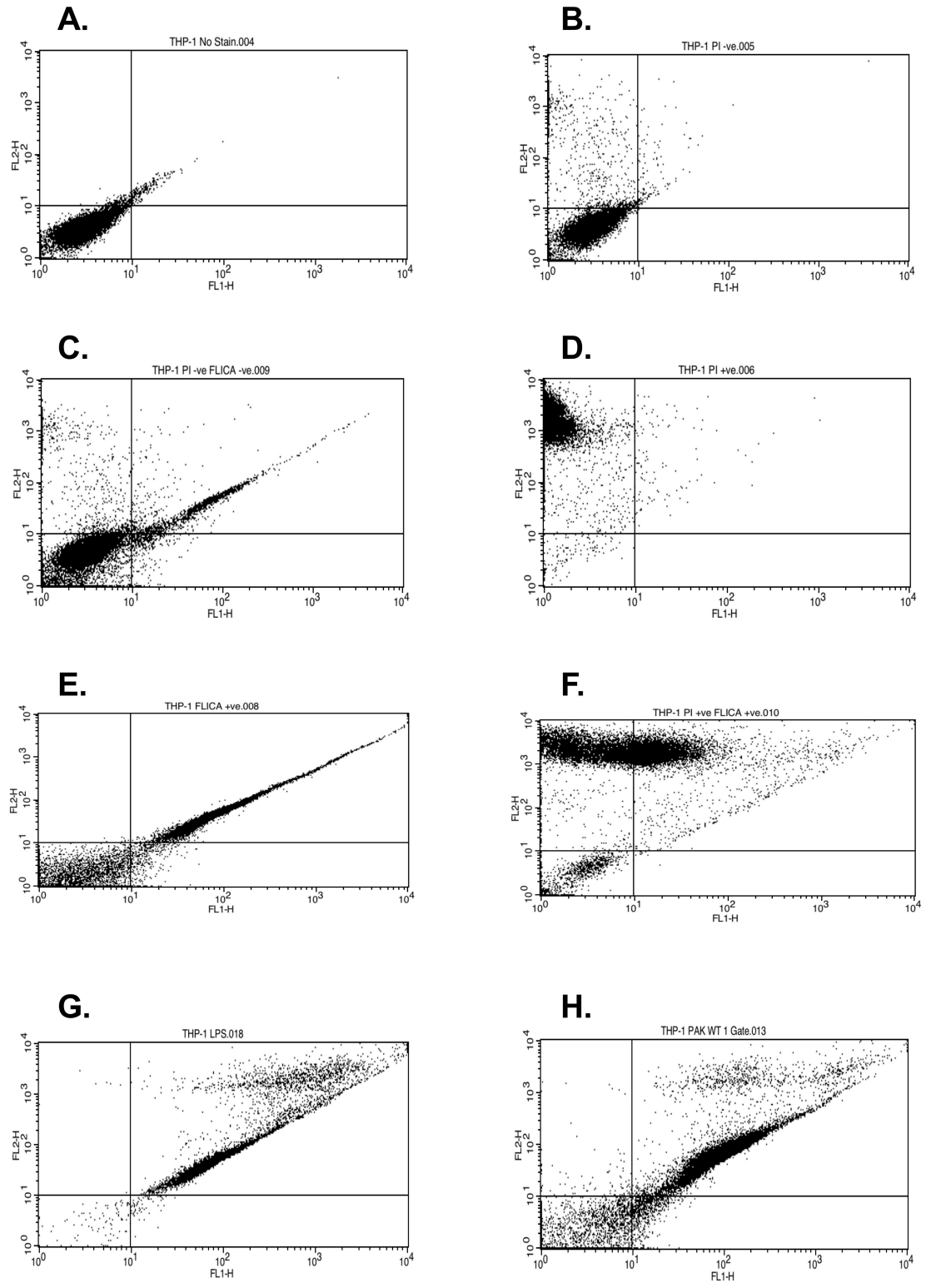


Figure 4


A.
THP-1 No Stain.004
B.
THP-1 PI -ve.005
C.
THP-1 PI -ve FLICA -ve.009
D.
THP-1 PI +ve.006
E.
THP-1 FLICA +ve.008
F.
THP-1 PI +ve FLICA +ve.010
G.
THP-1 LPS.018
H.
THP-1 PAK WT 1 Gate.013
FL1-H
FL2-H

Cotinue Figure 4

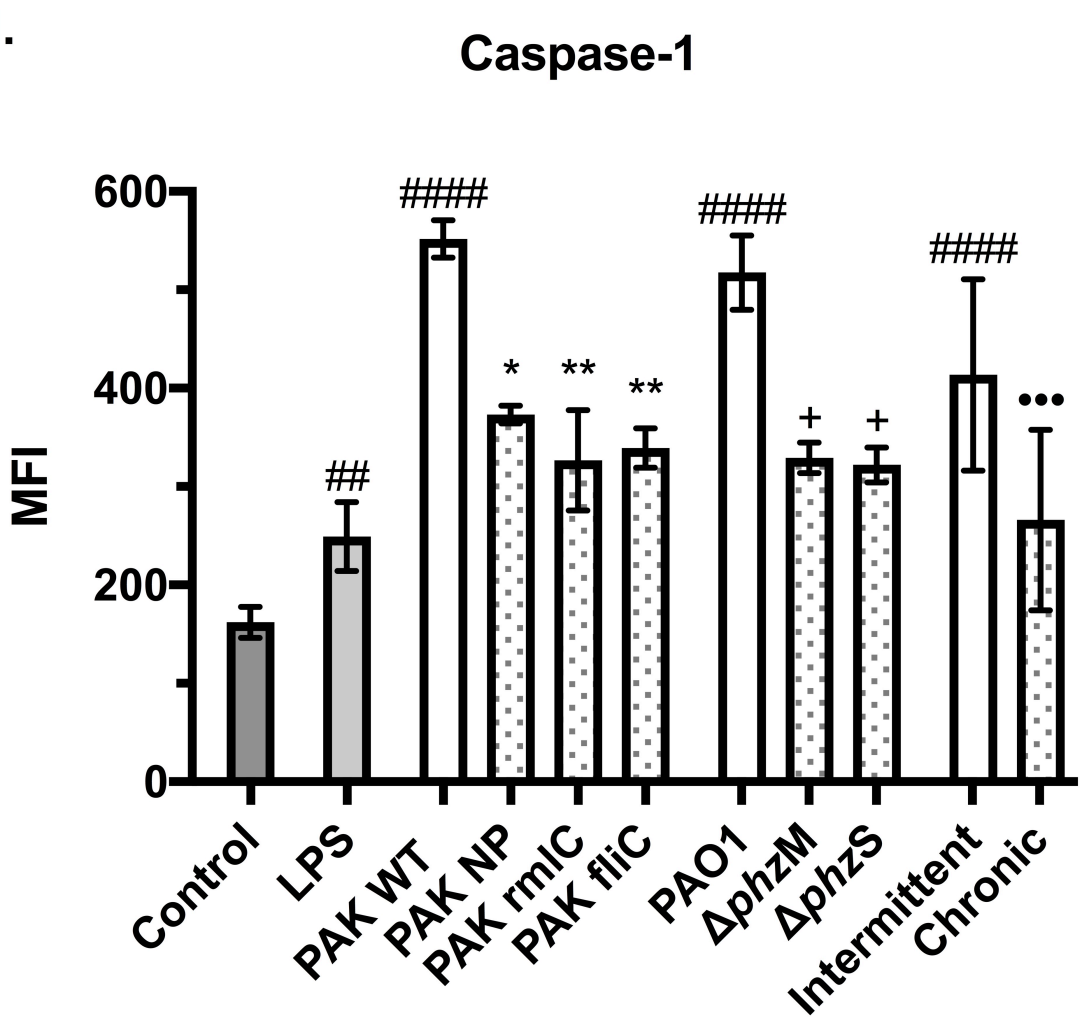
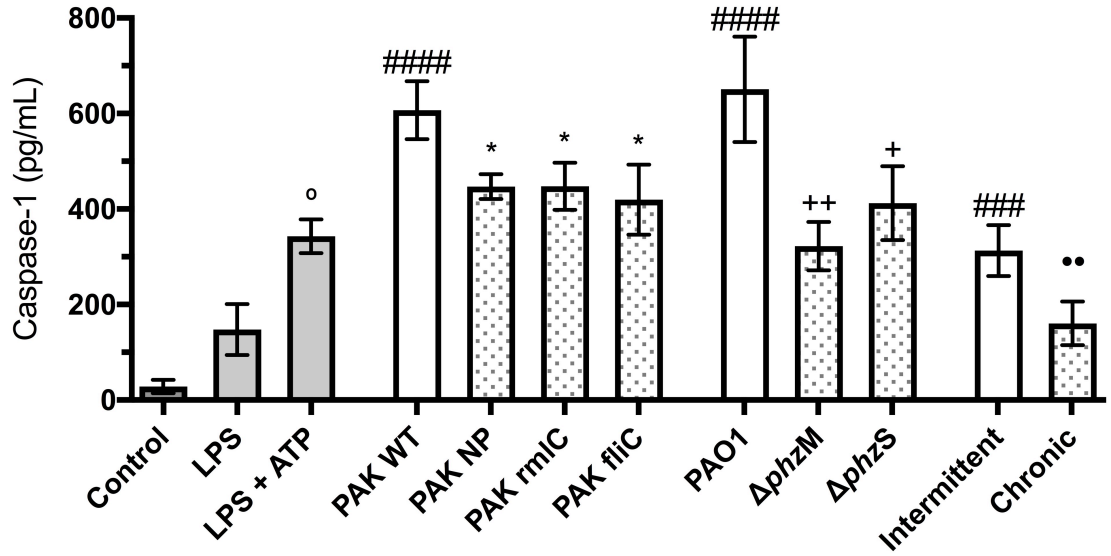


Figure 5

A.


Caspase-1 (pg/mL)
800
600
400
200
0
####
####
####
*
*
*
°
++
+
###
••
Control
LPS
LPS + ATP
PAK WT
PAK NP
PAK rmlC
PAK fliC
PAO1
ΔphzM
ΔphzS
Intermittent
Chronic

B.

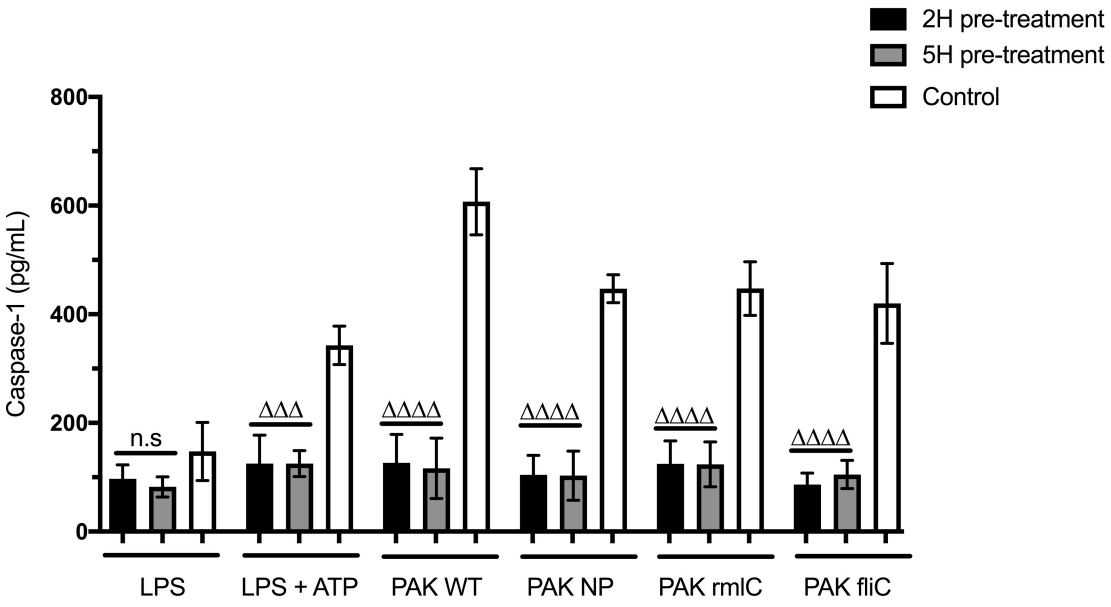

2H pre-treatment
5H pre-treatment
Control
Caspase-1 (pg/mL)
800
600
400
200
0
n.s
ΔΔΔ
ΔΔΔΔ
ΔΔΔΔ
ΔΔΔΔ
ΔΔΔΔ
LPS
LPS + ATP
PAK WT
PAK NP
PAK rmlC
PAK fliC

Figure 6

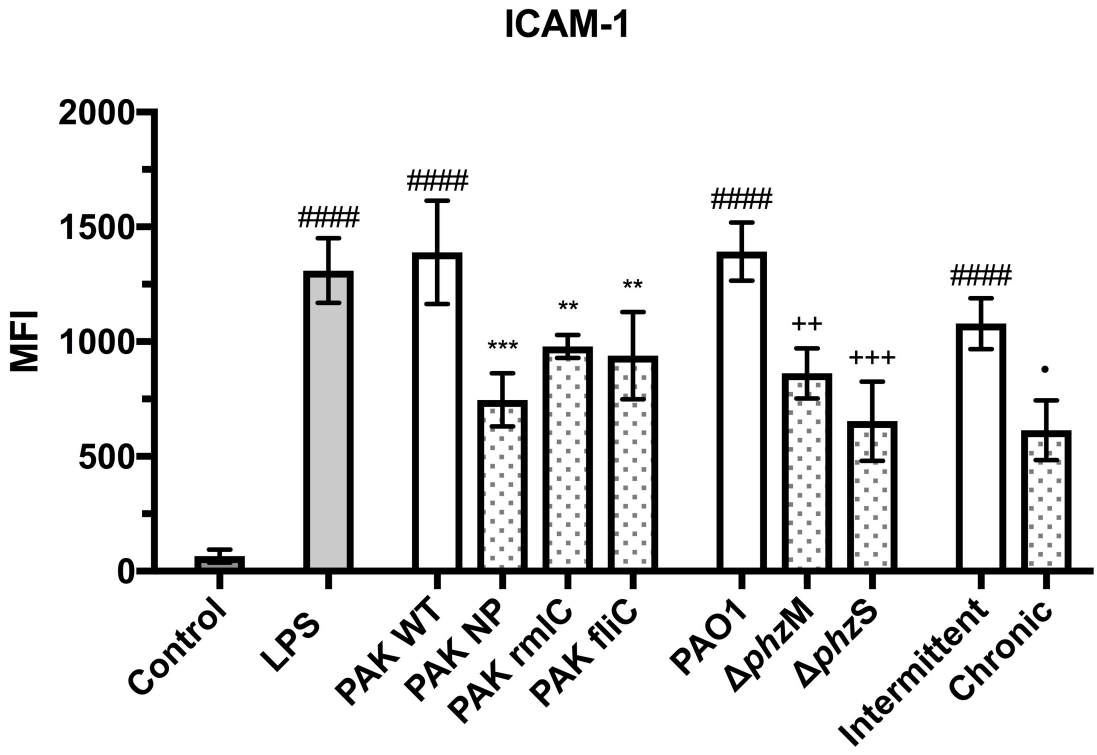
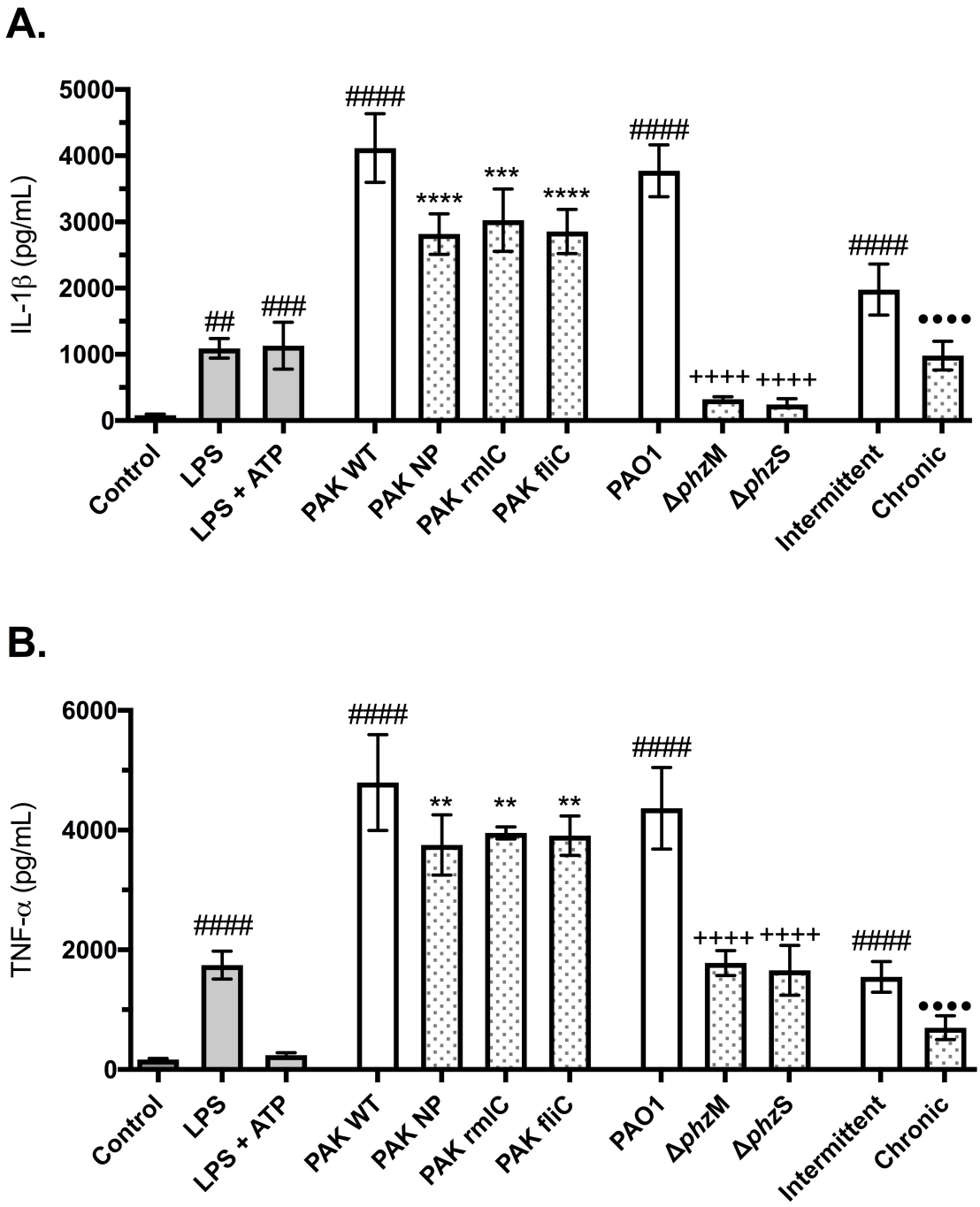

ICAM-1
MFI
2000
1500
1000
500
0
####
####
***
**
**
####
++
+++
####
Control
LPS
PAK WT
PAK NP
PAK rmlC
PAK fliC
PAO1
ΔphzM
ΔphzS
Intermittent
Chronic

Figure 7


A.
IL-1β (pg/mL)
5000
4000
3000
2000
1000
0
##
###
####
****
***
****
####
++++
++++
####
••••
Control
LPS
LPS + ATP
PAK WT
PAK NP
PAK rmlC
PAK fliC
PAO1
ΔphzM
ΔphzS
Intermittent
Chronic
B.
TNF-α (pg/mL)
6000
4000
2000
0
####
**
**
**
####
++++
++++
####
••••
Control
LPS
LPS + ATP
PAK WT
PAK NP
PAK rmlC
PAK fliC
PAO1
ΔphzM
ΔphzS
Intermittent
Chronic

Cotinue Figure 7

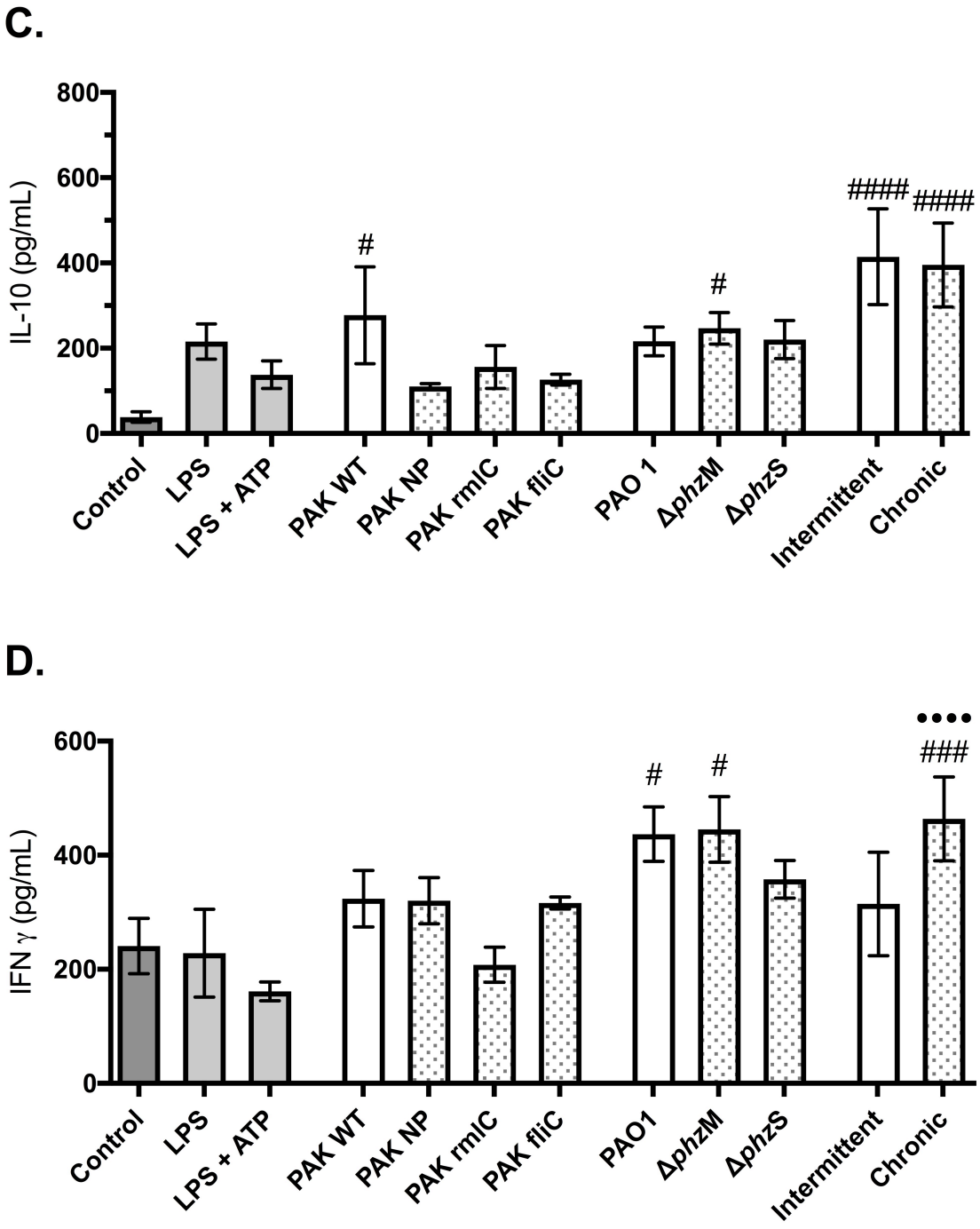
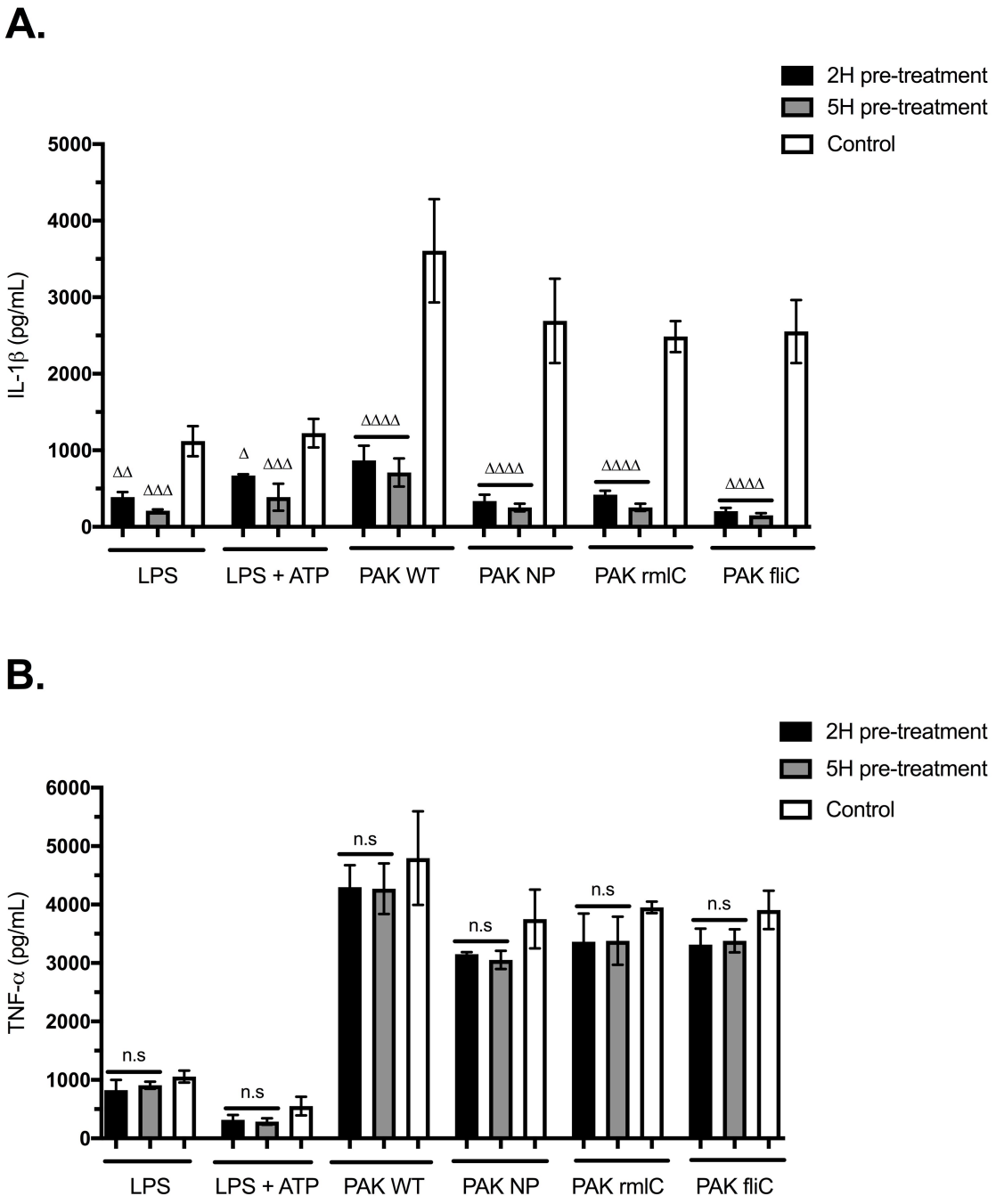


Figure 8


A.
2H pre-treatment
5H pre-treatment
Control
IL-1β (pg/mL)
5000
4000
3000
2000
1000
0
ΔΔ
ΔΔΔ
Δ
ΔΔΔ
ΔΔΔΔ
ΔΔΔΔ
ΔΔΔΔ
ΔΔΔΔ
LPS
LPS + ATP
PAK WT
PAK NP
PAK rmlC
PAK fliC
B.
2H pre-treatment
5H pre-treatment
Control
TNF-α (pg/mL)
6000
5000
4000
3000
2000
1000
0
n.s
n.s
n.s
n.s
n.s
n.s
LPS
LPS + ATP
PAK WT
PAK NP
PAK rmlC
PAK fliC

Chapter III: Syk inhibitor R406 down-regulates inflammation in an *in vitro* model of *Pseudomonas aeruginosa* infection

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Authors: Alaa Alhazmi, Joshua Choi, Marina Ulanova

Abstract

As *Pseudomonas aeruginosa* infections are characterized by strong inflammation of infected tissues anti-inflammatory therapies in combination with antibiotics have been considered for the treatment of associated diseases. Syk tyrosine kinase is an important regulator of inflammatory responses, and its specific inhibition was explored as a therapeutic option in several inflammatory conditions; however, this has not been studied in bacterial infections. We used a model of *in vitro* infection of human monocytic cell line THP-1 and lung epithelial cell line H292 with both wild type and flagella-deficient mutant of *P. aeruginosa* strain K, as well as with clinical isolates from cystic fibrosis patients, to study the effect of a small molecule Syk inhibitor R406 on inflammatory responses induced by this pathogen. One-hour long pretreatment of THP-1 cells with 10 μM R406 resulted in a significant down-regulation of the expression of the adhesion molecule ICAM-1, pro-inflammatory cytokines TNFα and IL-1β, and phosphorylated signaling proteins ERK2, JNK, p-38, and IκBα, as well as significantly decreased TNF-α release by infected H292 cells. The results suggest that Syk is involved in the regulation of inflammatory responses to *P. aeruginosa*, and R406 may potentially be useful in dampening the damage caused by severe inflammation associated with this infection.

Keywords: *Pseudomonas aeruginosa*, cystic fibrosis, Syk, small molecule inhibitor, R406, inflammation, cytokine

3.1 Introduction

Pseudomonas aeruginosa is the major cause of chronic pulmonary infection in cystic fibrosis (CF) patients as well as of other serious conditions in immunocompromised individuals [1-4]. *P. aeruginosa* is a Gram-negative opportunistic pathogen armed with potent virulence factors including the type III secretion and quorum sensing systems, lipopolysaccharide, several powerful exotoxins, and various enzymes that contribute to disease pathogenesis via severe tissue damage and inflammation as well as immune evasion [5]. As *P. aeruginosa* infection is characterized by exaggerated inflammatory responses, anti-inflammatory therapy is considered important for treatment of *P. aeruginosa*-associated conditions [6]. In particular, intracellular protein kinases involved in the regulation of pro-inflammatory signaling pathways may represent potential therapeutic targets. We have recently found that an inhibitor of Syk tyrosine kinase piceatannol is able to down-regulate inflammatory responses in *P. aeruginosa*-infected lung epithelial cells [7]. However, the effect of piceatannol in this model extended beyond inhibition of Syk, i.e. via potential modulation of Syk-independent signaling pathways [7]. A small molecule inhibitor, N4-(2,2-dimethyl-3-oxo-4H-pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine (R406) was demonstrated to selectively inhibit Syk kinase activity in an ATP-competitive manner both *in vitro* and *in vivo* [8-11]. R406 is the active metabolite of an orally available drug Fostamatinib, which had undergone several clinical trials for treatment of

some autoimmune and allergic diseases and hematological malignancies [12]. However, it is unknown whether R406 can modulate inflammatory responses in infections. In this study, we sought to assess the effect of R406 on inflammatory markers associated with *P. aeruginosa* infection of human monocytic and lung epithelial cells.

3.2 Materials and Methods

3.2.1 Cell culture conditions

The THP-1 human acute monocytic leukemia cell line (ATCC, Manassas, VA) was used at the passage numbers of 6-20. These cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat inactivated fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS) and 1% antibiotic-antimycotic (Invitrogen, Burlington, ON, Canada). Cells were grown at 37°C with 5% CO_2 and seeded every 3-4 days when cell counts neared 1×10^6 cells/mL. In preparation for experiments, the cells were centrifuged at 400 × g for 5 minutes, washed with sterile PBS (pH 7.4), and suspended in culture medium without antibiotics. To induce differentiation, THP-1 cells were plated at 1×10^6 cells/mL/well in 24-well plates (Costar, Corning Incorporated, Corning NY), in serum- and antibiotic-RPMI 1640 medium. Cells were then treated with 20 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich) at 37°C in 5% CO_2 for 12 hours, then washed and re-suspended in the same medium. After 48 hours of further incubation, the cells were washed twice with serum- and antibiotic-free medium and used for experiments.

The H292 human muco-epidermoid bronchiolar carcinoma cell line (ATCC) was used at the passage numbers of 10-25. These cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS without antibiotics. Cells were

grown at 37°C with 5% CO_2 and seeded every 3-4 days when confluency approached 80%. For viability testing, the cells were detached using 0.5% Trypsin-EDTA (Gibco, Eugene, OR), centrifuged at 400 × g for 5 minutes, washed with sterile PBS (pH 7.4), and suspended in culture medium. Cell viability was determined by the trypan blue exclusion method using a ViCell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA).

3.2.2 *Pseudomonas aeruginosa* strains and in vitro infectious model

Pseudomonas aeruginosa strain K wild type (PAK WT, provided by Dr. RJ Irvin, University of Alberta, Edmonton, AB) and the isogenic *P. aeruginosa* mutant PAK fliC (flagella deficient, provided by Dr. AS Prince, Columbia University, New York), as well as *P. aeruginosa* clinical isolates from sputum samples of CF patients were used (Table). One clinical isolate from an intermittently colonized and another from a chronically infected patient (the latter obtained during longitudinal observation at the Danish CF Center) were kindly provided by Dr. N Høiby (University Hospital Rigshospitalet, Copenhagen, Denmark). The characteristics of the isolates are described in our previous study [13].

The bacteria were maintained on Luria Burtani (LB) medium (Fischer Scientific, Fair Lawn, NJ) with 1% agar (LBA). A single colony of *P. aeruginosa* was grown overnight in sterile LB medium on a shaking platform at 150 rpm and diluted by a factor of 20 into fresh sterile LB medium. Cultures were allowed to grow for approximately 1 hour, until mid-log phase when optical density at 600 nm (OD_{600}) reached 0.30. The culture was then centrifuged at 3,500 × *g* for 20 minutes at 4°C and washed twice in PBS. Following the final re-suspension, bacteria were diluted to an OD_{600} of 0.30 in RPMI 1640 that corresponded to approximately 2×10^8 CFU/mL, as determined by serial

dilutions and drop plating on LBA. From this stock, bacteria were added to either H292 cells to obtain a multiplicity of infection (MOI) of 50, as was optimized in our previous experiments [7], or THP-1 cells at a MOI of 5. The latter conditions were optimized using THP-1 cells infected with PAK WT during 1, 2, 6, 12, or 18 hours at MOI of 1, 5, or 10.

3.2.3 Stimulation of THP-1 cells via Fcγ-receptor cross-linking

The 96-well plates (Falcon, Corning Incorporated) were coated with human IgG (Sigma-Aldrich) at concentrations of 10 and 100 μg/mL and incubated for 3 hours at 37°C, followed by overnight incubation at 4°C, then the plates were washed twice with sterile PBS. THP-1 cells at concentration of 0.4×10^6 cells/mL in 200 μL were added to the coated wells and incubated for 18 hours at 37°C with 5% CO_2.

3.2.4 Pretreatment with R406

THP-1 or H292 cells were grown for 24 hours to 0.4×10^6 cells/mL, or until they reached approximately 80% confluence, respectively, and R406 (AstraZeneca) dissolved in DMSO was added to the medium to achieve a final concentration of 10 μM. The cells were incubated in the presence of R406 for 1 hour, then washed once with PBS and used for experiments. These conditions were developed based on published literature describing R406 pretreatments [9,10,14] and our cellular viability testing using R406 concentrations of 1, 5, 10, 15, and 20 μM. No noticeable effect of R406 concentrations up to 10 μM on cell viability tested during one hour was detected (97-99% viable cells), nor significant decrease in either THP-1 or H292 cell viability following 18-hour-long incubation with 10 μM of R406 occurred. Viability of THP-1 following 18 hour-long incubation with R406 or without R406 was 82% and 75% ($P>0.05$), for H292 cells, it was 84% and 81%, correspondingly ($P>0.05$).

3.2.5 Flow cytometry analysis of ICAM-1 expression

THP-1 cells (0.4×10^6) were infected with PAK WT for 6 hours at 37°C, 5% CO2, then washed and re-suspended in 100 µL of 0.1% BSA-PBS containing PE-conjugated mAb against ICAM-1 (Mouse anti-human CD54, BD Pharmigen, Mississauga, ON) at a dilution of 1:50 and incubated for 1 hour at 4°C. Following incubation, cells were washed twice with PBS and analyzed by flow cytometry on the FACSCailbur (BD Bioscience, Mississauga, ON, Canada). The data were analyzed using CellQuest Pro software and expressed as mean fluorescence intensity (MFI).

3.2.6 ELISA for cytokine detection

To measure the release of cytokines, PMA-differentiated THP-1 or H292 cells were infected with *P. aeruginosa* (MOI of 10, or 50, correspondingly), for 1 hour at 37˚C, with 5% CO_2, and then 100 µg/mL gentamicin was added, followed by incubation for a further 17 hours at 37°C with 5% CO2. Following stimulation, cell culture supernatants were collected and stored at -80°C until analysis. The levels of TNFα and IL-1β were measured using eBioscience Ready-Set-Go ELISA kits (San Diego, CA) according to the manufacturer's protocol. The lower detection limits of the assays were 2 pg/mL for IL-1β and 4 pg/mL for TNFα. Samples from three independent experiments were run in triplicate.

3.2.7 Immunoprecipitation and Western blot

THP-1 cells (2×10^6) were infected with PAK WT at an MOI of 5 for 2 hours at 37°C, 5% CO_2. Following stimulation, the cells were centrifuged, washed, re-suspended in 100 µL of ice-cold RIPA lysis buffer, and incubated for 30 minutes at 4 °C. Following incubation, the cells were centrifuged at 8,000 × g for 10 minutes and protein lysate was

collected. Isolated proteins were immunoprecipitated with polyclonal anti-Syk antibody (N-19) (Santa Cruz Biotehnology, CA) using magnetic Protein A beads (Bio-Rad, Hercules CA), according to the manufacturer's protocol. Samples were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, probed with primary antibody, i.e. anti-phospho-tyrosine (P-Tyr-100) (Cell Signaling Technology), or monoclonal anti-syk antibodies (4D10) (Santa Cruz Biotechnology, CA) followed by HRP-conjugated secondary antibody (7074S) (Cell Signaling Technology), and developed using enhanced chemiluminescence. Bands were scanned and images analyzed using ChemiDoc XRS (Bio-Rad). For analysis of total and phosphorylated intracellular signaling proteins, THP-1 cells were stimulated with PAK WT (MOI of 5), for 15, 30, or 60 minutes at 37°C. Protein lysates were collected and stored at -80 °C. For analysis of protein expression by Western blot we used: monoclonal anti-JNK (D-2), anti-phospho JNK (G-7), anti-ERK 2 (12A4), anti-phospho ERK 2 (E-4), anti-p38α (9F12), anti-phospho p38α (E-1), anti-IκB-α (H-4), anti-phospho IκB-α (B-9), anti- β-actin (C4) and mouse IgGκ BP-HRP (Santa Cruz Biotechnology). In some cases, the blots were stripped and re-probed with other antibodies.

3.2.8 Statistical analysis

All the experiments were repeated at least 3 times. Data were expressed as mean +/- SEM for n independent experiments. For comparison of two sample means, Student's t test was applied. GraphPad Prism 7.0 (La Jolla, CA, USA) was used for the analysis. P -values <0.05 were considered significant.

3.3 Results:

3.3.1 R406 down-regulates ICAM-1 expression induced by *P. aeruginosa* infection

As the intercellular adhesion molecule 1 (ICAM-1) typically becomes up-regulated during inflammatory responses, particularly in cells infected with *P. aeruginosa* [15] we tested the effect of R406 on the cell-surface expression of ICAM-1 in THP-1 cells exposed to a virulent *P. aeruginosa* strain K (PAK WT) at an MOI of 5. As shown on Figure.1A, 6-hour long infection resulted in >15-fold increase in ICAM-1 mean fluorescence intensity (MFI) compared to uninfected cells. One hour-long pre-incubation of THP-1 cells with R406 used in concentrations between 0.1 and 20 μM down-regulated ICAM-1 expression in a dose-dependent manner, with a statistically significant effect for all the R406 concentrations >0.5 μM (Fig. 1A). There was no noticeable effect of R406 concentrations up to 10 μM on cell viability; the percentage of viable cells after one hour of incubation with R406 was 97-99% (data not shown). Likewise, one hour-long incubation of THP-1 cells with 1, 5, or 10 μM of R406 did not have any visible effect on the baseline ICAM-1 expression (data not shown).

To confirm the effect of R406 on Syk in our model, we stimulated THP-1 cells via Fcγ-receptor (FcγR) cross-linking, which is known to induce Syk-dependent signaling [16]. While stimulation of THP-1 cells with immobilized human IgG resulted in a significant increase in ICAM-1 surface expression in a dose-dependent manner, pre-treatment with 10 μM R406 caused an attenuation of ICAM-1 expression at both 10 and 100 μg/mL IgG concentrations ($P<0.05$) (Figure. 1B). Moreover, tyrosine phosphorylation of Syk induced by two hour-long exposure of THP-1 cells to live bacteria was significantly down-regulated in cells, pre-treated with 10 μM R406 (Figure.

1C). These experiments imply that down-regulation of ICAM-1 in *P. aeruginosa* infected cells by R406 could be mediated by inhibition of Syk-mediated signaling.

3.3.2 R406 down-regulates the release of pro-inflammatory cytokines TNFα and IL-1β induced by *P. aeruginosa* infection

To further test the effect of R406 in our model, we studied the release of cytokines TNFα and IL-1β, which are the hallmarks of inflammatory responses caused by *P. aeruginosa* infection, by using a virulent *P. aeruginosa* strain K (PAK WT), the isogenic *P. aeruginosa* mutant PAK fliC (flagella-deficient), and clinical isolates from two CF patients (intermittently colonized and chronically infected). One hour-long infection of differentiated THP-1 cells, or H292 cells with PAK WT, followed by adding gentamicin with further 17 hours of incubation resulted in a large TNFα release by both cell types, with THP-1 cells producing over 35-fold greater amount of this cytokine as compared to H292 cells (Figure. 2B-C).

When flagella-deficient mutant (PAK fliC) was used for stimulation, TNFα release by both cell lines was lower compared to stimulation with PAK WT ($P<0.01$) Interestingly, although stimulation of THP-1 cells with either clinical *P. aeruginosa* isolate resulted in lower TNFα release ($P<0.0001$), in case of H292 cells, an isolate from a chronically infected CF patient induced higher TNFα release compared to PAK WT ($P<0.001$), PAK fliC ($P<0.0001$), and isolate from a CF patient with intermittent *P. aeruginosa* infection ($P<0.001$). Nevertheless, TNFα release was significantly decreased in all infected cell cultures pretreated with R406, except for THP-1 cells stimulated with an isolate from a chronically infected CF patient (Figure. 2B-C).

While unstimulated differentiated THP-1 cells only produced a low amount of IL-1β (78 ±9 pg/mL), infection with PAK WT resulted in a large increase in IL-1β release (3993 ±245 pg/mL, P<0.0001). Stimulation with PAK fliC, or either isolate from a CF patient with intermittent or chronically *P. aeruginosa* infected also significantly upregulated IL-1β release, although to a lesser degree compared to PAK WT (P<0.0001, P<0.01, and P<0.05 respectively). The lowest amount of IL-1β (1310 ±281 pg/mL) was released by THP-1 cells stimulated with an isolate from a chronically infected CF patient (significant lower than following stimulation with PAK WT, P<0.0001). Despite of different degrees of IL-1β release induced by *P. aeruginosa* strains, pretreatment of differentiated THP-1 cells with R406 down-regulated this response (Figure. 2A).

3.3.3 R406 down-regulates the expression of phosphorylated ERK2, JNK, p-38, and IκBα in *P. aeruginosa* infected THP-1 cells

As intracellular signaling molecules ERK2, JNK, p-38, and IκBα have been recognized as important regulators of inflammatory responses induced by *P. aeruginosa* [17-19], we investigated the effect of R406 on the expression of these total and phosphorylated proteins in our model. Stimulation of THP-1 cells with PAK induced significant up-regulation of phosphorylated ERK2 at 30 min (P<0.01) and 60 min (P<0.01), JNK at 15 min (P<0.001), 30 min (P<0.001), and 60 min (P<0.001), p38 at 15 min (P<0.001), 30 min (P<0.0001), and 60 min (P<0.0001), and IκBα at 30 min (P<0.01) and 60 min (P<0.001). Pretreatment of infected cells with R406 led to a decreased expression of all phosphorylated signaling molecules that was statistically significant for JNK and p-38 at 15, 30, and 60 minutes of stimulation, and for IκBα and ERK2 at 30 and 60 minutes of stimulation (Figure. 3A-D).

3.4 Discussion

This study shows that a small molecule inhibitor of Syk down-regulates inflammatory responses of human cells infected with *P. aeruginosa*. Specifically, in monocytic cell line THP-1, R406 caused a significant decrease in cell surface expression of ICAM-1, an adhesion molecule, which mediates leukocyte migration to inflammatory sites, in a dose-dependent manner, as well as down-regulated the release of pro-inflammatory cytokines TNFα and IL-1β. The transcriptional regulation of all these three molecules is largely dependent on the activation of transcription factor NF-κB, which is known to be a downstream target of Syk-mediated signaling along with the MAPK cascade [16,20]. Indeed, R406 caused a decrease in the expression of phosphorylated ERK2, JNK and p-38, as well as of IκBα; the latter, when phosphorylated, facilitates nuclear translocation of NF-κB, which is required for its activation and resulting production of inflammatory mediators [21]. In our previous study, inhibition of Syk using small interfering RNA caused down-regulation of the MAPK cascade phosphorylation and nuclear translocation of p65 NF-κB induced by TNFα stimulation of lung epithelial cells [22]. The data of the present study extend our earlier observations to monocytic cells and indicate that Syk is involved in the regulation of pro-inflammatory responses to *P. aeruginosa* infection via activation of downstream signalling pathways, including MAPK-mediated one. In support of this idea, we found an increase in the expression of tyrosine-phosphorylated Syk, an indicator of Syk activation, following two hour-long *P. aeruginosa* infection, and a decrease in the expression of phospho-Syk following pre-treatment with R406 (Figure. 1C). As release of mature IL-1β requires inflammasome activation, in addition to IL-1β gene transcription, the effect of R406 on IL-1β release

suggests Syk involvement in the regulation of inflammasome activation in our model [23]. This is not surprising as previous studies identified Syk as a key mediator of NLRP3 inflammasome activation and IL-1β secretion in innate immune cells stimulated with fungi and crystals [24-28].

There are potentially multiple pathways of Syk activation during *P. aeruginosa* infection of monocytic cells. This non-receptor protein tyrosine kinase is best known as a critical component of immunoreceptor tyrosine-based activation motifs (ITAM)-dependent signaling in hematopoietic cells involving Fc receptors, T-, B-, and NK cell receptors [29]. Congruently, in our experiments, we observed a strong inhibitory effect of R406 on ICAM-1 expression induced by a classical mechanism of Syk activation, i.e. via Fcγ receptor cross-linking, with ICAM-1 expression level decreased to the baseline while using a 10 μg/mL concentration of human IgG for receptor activation (Figure. 1B). However, none of the cellular responses to *P. aeruginosa* have been completely inhibited by R406, although we could achieve their significant down-regulation using a concentration of 10 μM, which was commonly used in studies by others [9]; in case of ICAM-1, lower concentrations of 0.5 to 5 μM were also effective (Figure 1A). The data suggest that although Syk is certainly involved in the regulation of inflammatory responses to *P. aeruginosa* infection, it does not represent the major pathway among multiple mechanisms operating in cellular responses to this highly virulent microorganism, which is capable to interact with many pathogen-recognition receptors, including Toll-like receptors, Nod-like receptors, integrins, C-type lectins, asialoGM1, *etc* [15,30,31]. Ability of *P. aeruginosa* to stimulate TNFα and IL-1β synthesis and release from human monocytes, and activation of transcription factors NF-κB and AP in

infected cells have been established by previous studies [17,32-34]. Syk involvement in the regulation of signals generated by the engagement of TLR-4 complex by its ligand LPS in human neutrophils and macrophages has also been previously demonstrated [35-37], and this mechanism likely operates in our model. Recent studies expanded our understanding of the role of Syk in fine-tuning of cellular responses stimulated by the engagement of innate immune receptors [38,39]. For example, it was demonstrated that in macrophages and dendritic cells, Syk regulates TNFα exocytosis induced by stimulation of TLR9 by bacterial CpG DNA [40]; such mechanism may potentially be involved in responses of differentiated THP-1 cells to *P. aeruginosa*. In addition, innate immune responses activated by *P. aeruginosa* result in the amplification of inflammatory responses, as for example, TNFα further activates the inflammatory cascade via its own receptor associated signaling [41]. The complexity of cellular responses to *P. aeruginosa* is further augmented by cross talk among multiple signalling pathways, including both pro- and anti-inflammatory [42].

Syk may become activated following *P. aeruginosa* infection via several potential mechanisms. It is well recognized that Syk is significantly involved in several ITAM-independent signalling pathways, which are mediated by its interaction with G-protein coupled receptors, pattern recognition, and cytokine receptors [43,44]. In particular, Syk can be activated via interaction with integrin receptor cytoplasmic domains that is especially significant in lung epithelial cells, which do not express the plethora of innate immune receptors typical for leukocytes [45]. Our previous research demonstrated the involvement of integrin receptors in *P. aeruginosa* internalization and recognition by A549 alveolar epithelial cells; moreover, the data suggested an important role of integrin-

mediated signaling in inflammation induced by this infection [46]. In the present study, the release of TNFα by infected bronchiolar epithelial cells was significantly down-regulated by R406 implicating the involvement of Syk-dependent signaling in inflammatory responses to *P. aeruginosa* by lung epithelial cells, in addition to monocytes (Figure 1C). Indeed, we have previously demonstrated that H292 cells express Syk [7]; however, it is uncertain whether or not Syk is exclusively engaged via integrin receptors in this particular cell line, or some other mechanisms, for example, those mediated by TNF-receptor signaling are involved [47].

Because Syk combines both kinase and adaptor protein properties, this molecule is capable to interact with multiple protein targets, and this explains why its inhibition leads to numerous biological effects. Indeed, Syk has been considered as a target for therapy of such diverse conditions as allergic diseases, rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, and B-cell lymphoma, with several pharmacological compounds undergoing clinical trials [12]. One potential application could be the use of Syk inhibitors to dampen severe pro-inflammatory responses associated with pulmonary *P. aeruginosa* infection, which affects CF patients, as well as occurs in ventilator-associated pneumonia, aggravates the course of chronic obstructive pulmonary disease (COPD), and causes severe complications in cancer patients with neutropenia, caused by chemotherapy that predisposes to *P. aeruginosa* pneumonia [1-4]. In our previous study, we found that a natural Syk inhibitor piceatannol significantly suppressed inflammation, oxidative stress, apoptosis, and bacterial internalization in a model of *P. aeruginosa* infected pulmonary epithelial cells, although not all of these outcomes could be attributed to Syk-specific effect [7]. Results of the current study

corroborate our previous observations using this time both a model of infected THP-1 cells, which represent innate immune cells, and a bronchiolar epithelial cell line H292 [48].

As bronchiolar epithelial cells represent the major component of the airway lining, have receptors for *P. aeruginosa*, are the site of infection, generate inflammatory responses to this infectious agent, and express Syk, they could be the major targets for potential therapeutic intervention using Syk inhibitors. Importantly, the response of H292 cells to stimulation with various strains of *P. aeruginosa* was noticeably different from the response by differentiated monocytic THP-1 cells (Figure. 2B-C). Although the release of TNFα by H292 cells infected with PAK WT or PAK fliC was approximately 50-times lower than the one by THP-1 cells, infection with clinical *P. aeruginosa* isolates caused relatively higher TNFα production in H292 cells. In particular, H292 cells infected with *P. aeruginosa* of a CF patient with long-term chronic infection released the largest amount of TNFα in comparison to other *P. aeruginosa* strains, i.e. 202 ±5 pg/mL, although THP-1 cells produced significantly less TNFα when stimulated with either clinical isolate (1545 ±237 pg/mL and 714 ±102 pg/mL) as compared to both wild-type (4795 ±463 pg/mL) and flagella-deficient (3691 ±255 pg/mL) laboratory strains PAK WT. These data corroborate our previous observations that *P. aeruginosa* isolates from chronically infected CF patients have increased abilities of causing inflammatory responses of A549 alveolar epithelial cells in comparison to bacteria from patients with intermittent *P. aeruginosa* colonization, owing to the adaptation process in the CF long during long-term infectious process [13]. The isolate #19731A/92 was obtained from a CF patient with 18-year long chronic *P. aeruginosa* infection [13]. As a flagella-deficient

strain (PAK fliC) induced significantly lower release of cytokines TNFα and IL-1β compared to the wild-type bacteria (Figure 2A-C) these data emphasize importance of flagella in stimulating potent pro-inflammatory responses to *P. aeruginosa* infection via the activation of pattern-recognition receptors such as TLR5 and NLRC4 inflammasome [49,50]. Importantly, in bronchiolar epithelial cells, R406 was able to significantly down-regulate TNFα release caused by *P. aeruginosa* isolates from both chronically infected and intermittently colonized CF patients, although to a lesser degree than when the inhibitor was applied to cells, stimulated with PAK WT or PAK fliC (Figure 2C) suggesting potential clinical application of this inhibitor. However, as recent studies found that Syk is essential for flagellin-specific T cell responses, it is important to consider complexity of the regulatory role of this signaling molecule in immune responses [51].

Compared to an early used inhibitor piceatannol, R406 has been demonstrated to be much more selective for Syk. However, R406 is not entirely specific to Syk, and able to inhibit JAK2 in addition to Syk of similar potency [52]. Although the present findings suggest Syk involvement in the regulation of *P. aeruginosa* triggered inflammatory responses in both human monocytic and bronchiolar epithelial cells, it will be highly desired to test more specific Syk inhibitors. However, creating a truly selective Syk inhibitor apparently represents a challenge; indeed, all of the existing compounds with Syk-inhibitory capacities, including the most recent ones express certain off-target specificity [53,54]. When Fostamatinib, of which R406 is the active metabolite, was tested in phase II-III clinical trials for rheumatoid arthritis, adverse events related to its off-target effect have been noticed [55]. As it was demonstrated that inhibition of JAK2

down-regulated inflammatory responses in an animal model of polymicrobial sepsis, certain off-target effects of R406 may potentially be beneficial in case of *P. aeruginosa* infection [56]. Conducting clinical trials to ascertain capacity of this Syk inhibitor to alleviate exaggerated inflammatory responses, which significantly contribute to the pathogenesis of *P. aeruginosa* pulmonary infections may represent a sensible approach.

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Table

Table 1 Strains and Clinical Isolates of *P. aeruginosa* used in this study.

Strain/Isolate	Source/Reference
Wild-type PAK (PAK WT)	R.J. Irvin/Pasloske et al. (1985)
Flagella-deficient PAK (PAK fliC)	A.S. Prince/Feldman et al. (1998)
Isolate from intermittently colonized CF patient (9793/92)	Danish CF Centre/Hawdon et al. (2010)
Isolate from chronically infected CF patient (19731A/92)	Danish CF Centre/Hawdon et al. (2010)

Figure Legend

Figure 1 The effect of R406 on ICAM-1 expression induced by *Pseudomonas aeruginosa* strain K (PAK WT) infection, or Fcγ receptor (FcγR) cross-linking.

a) One hour-long pre-treatment of THP-1 cells with various concentrations of R406 decreased ICAM-1 expression in THP-1 cells infected with PAK at an MOI of 5 for 6 hours in a dose-dependent manner. **b)** Pre-treatment of THP-1 cells with 10μM R406 for 1 hour prior to their stimulation with immobilized human IgG at concentrations of 10 and 100 μg/mL decreased up-regulation of ICAM-1. THP-1 cells were infected with PAK or stimulated via FcγR cross-linking as described in Materials & Methods, and ICAM-1 surface expression determined using immunostaining and flow cytometry analysis. Data are expressed as mean fluorescence intensity (MFI). Results represent the mean ± SEM of 3 independent experiments; ###P<0.001, difference between un-stimulated and stimulated cells; *P < 0.05, **P<0.01, ***P<0.001, difference between stimulated R406 treated vs. un-treated cells. **c)** Ratios of Western blotting band intensity of Syk phosphorylated on tyrosine to total Syk. The lanes from left to right: un-stimulated THP-1 cells, THP-1 cells infected with PAK for 2 hours, THP-1 cells pretreated with 10μM R406 followed by infection with PAK WT. The bands of Syk and phosphotyrosine on immunoprecipitated Syk were detected at 72 kDa. Results represent 3 independent experiments. *** P < 0.001, difference between R406-treated and un-treated infected THP-1 cells.

Figure 2 The effect of R406 pre-treatment on cytokine expression induced by *Pseudomonas aeruginosa*.

Differentiated THP-1 cells were infected with *P. aeruginosa* for 1 hours at an MOI of 10 per 1×10^6 cells, and cultured for another 17 h in the presence of 100 μg/mL gentamicin. Unstimulated differentiated THP-1 cells (1×10^6) in complete culture medium served as a negative control. The supernatant was collected and IL-1β (**A**) and TNFα (**B**) concentrations (pg/mL) in culture supernatants were examined using ELISA. For TNFα expression by H292 cells (**C**)**,** the cells were infected at an MOI of 50 for 1 h and further cultured as described above. In samples involving R406, cells were pre-treated with 10 μM R406 for 1 hour prior to infection. Results represent the mean ± SEM of 3 independent experiments; * P < 0.05, **P <0.01, *** P < 0.001, ****P <0.0001, difference between R406-treated and un-treated infected cells.

Figure 3 The effect of R406 on expression of phosphorylated and total intracellular signaling proteins.

THP-1 cells were stimulated with *P. aeruginosa* strain K (PAK WT) at an MOI of 5 for 15, 30, or 60 minutes. Following stimulation, the levels of total and phosphorylated ERK2 (42 kDa), JNK (46 kDa), IκBα (41 kDa), and p-38 (38 kDa) were determined in cellular lysates by Western blot. Results are expressed as ratios of phosphorylated/total protein band intensity. In samples involving R406, cells were pre-treated with 10μM R406 for 1 hour prior to infection. β-actin served as a loading control. Results represent the mean ± SEM of 2 independent experiments; * P < 0.05, **P <0.01, *** P < 0.001, ****P <0.0001, difference between R406-treated and un-treated infected cells.

Figures

Figure 1

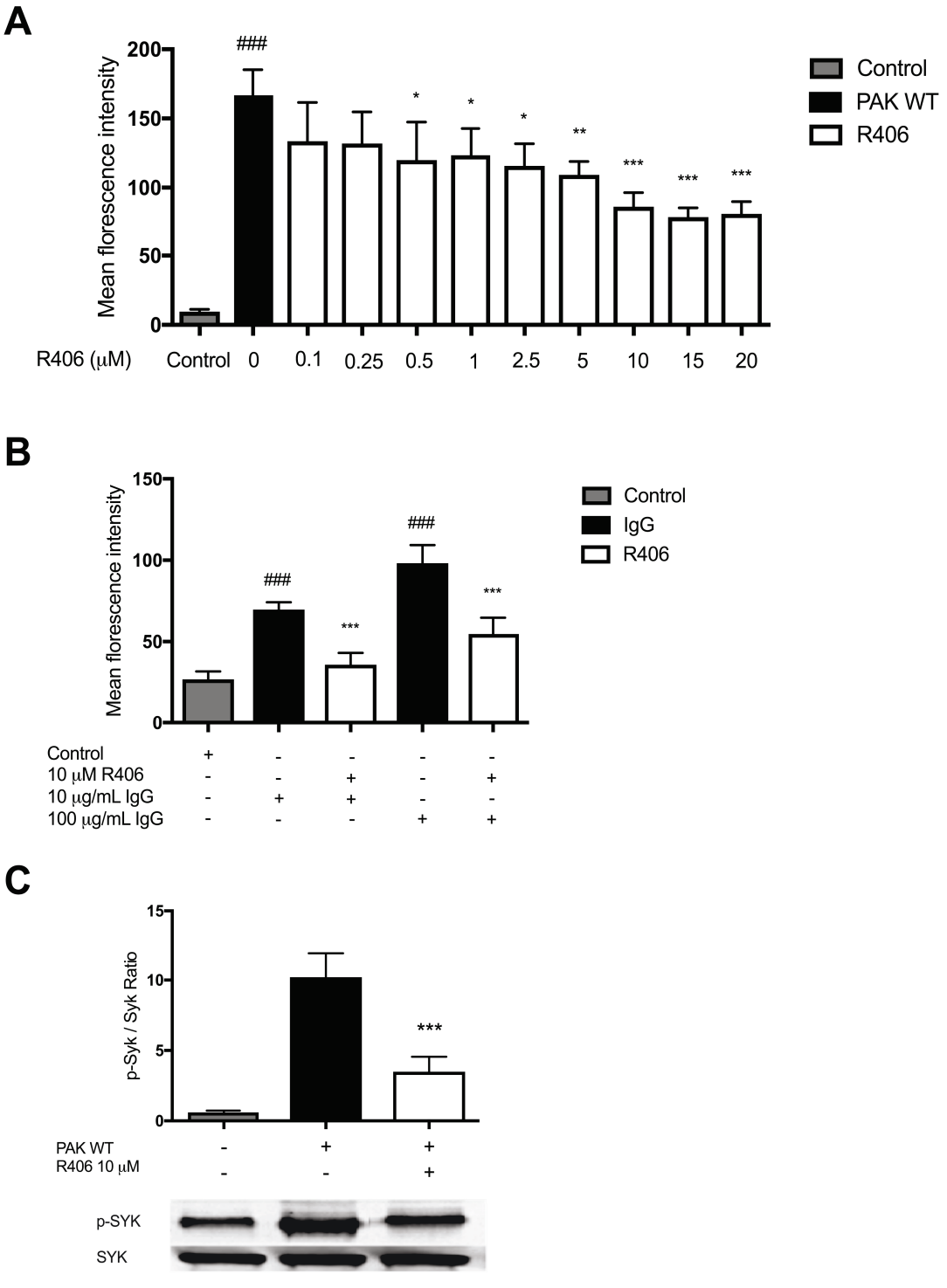
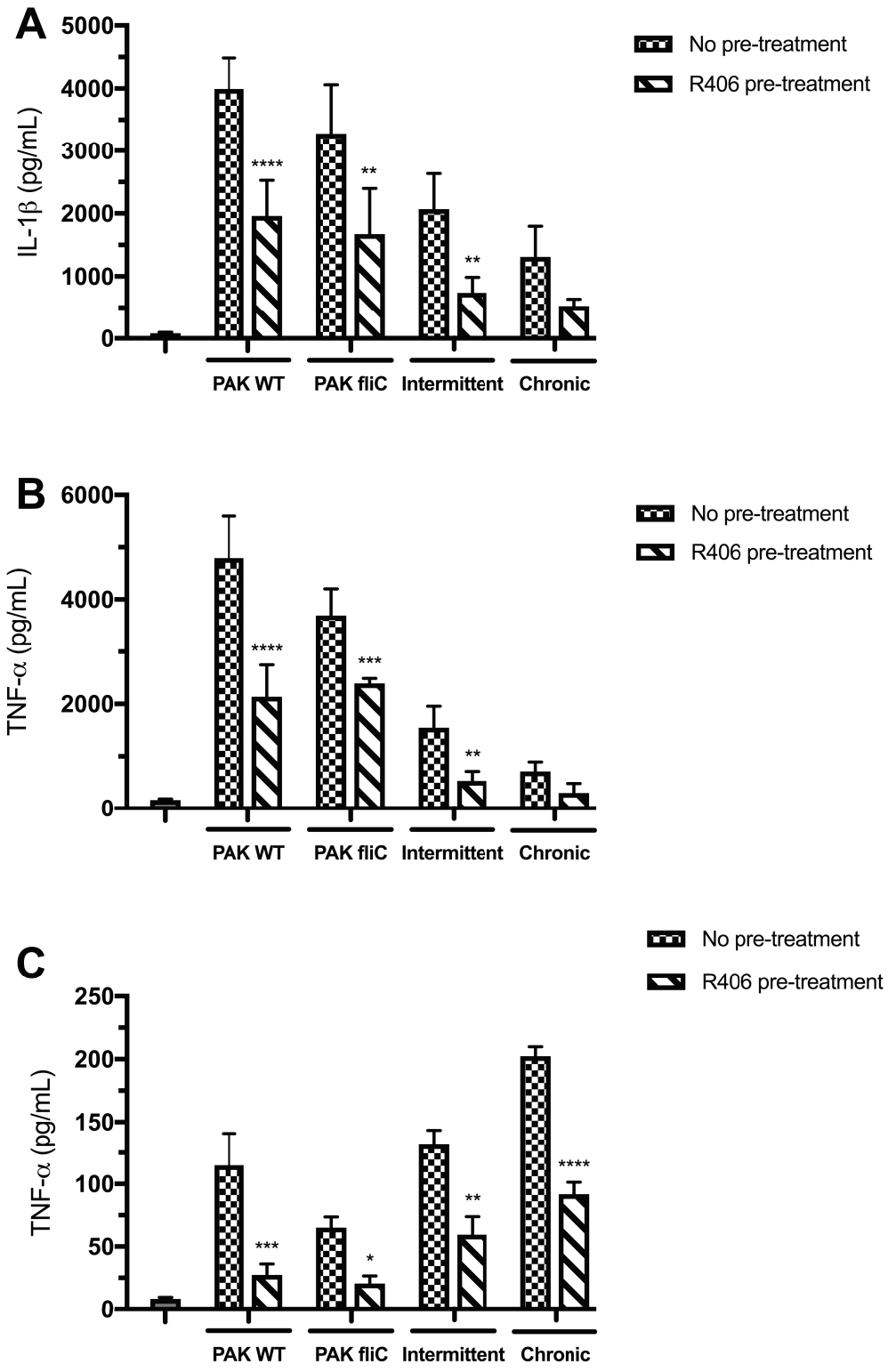


Figure 2


A
5000
4000
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IL-1β (pg/mL)
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No pre-treatment
R406 pre-treatment
PAK WT
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No pre-treatment
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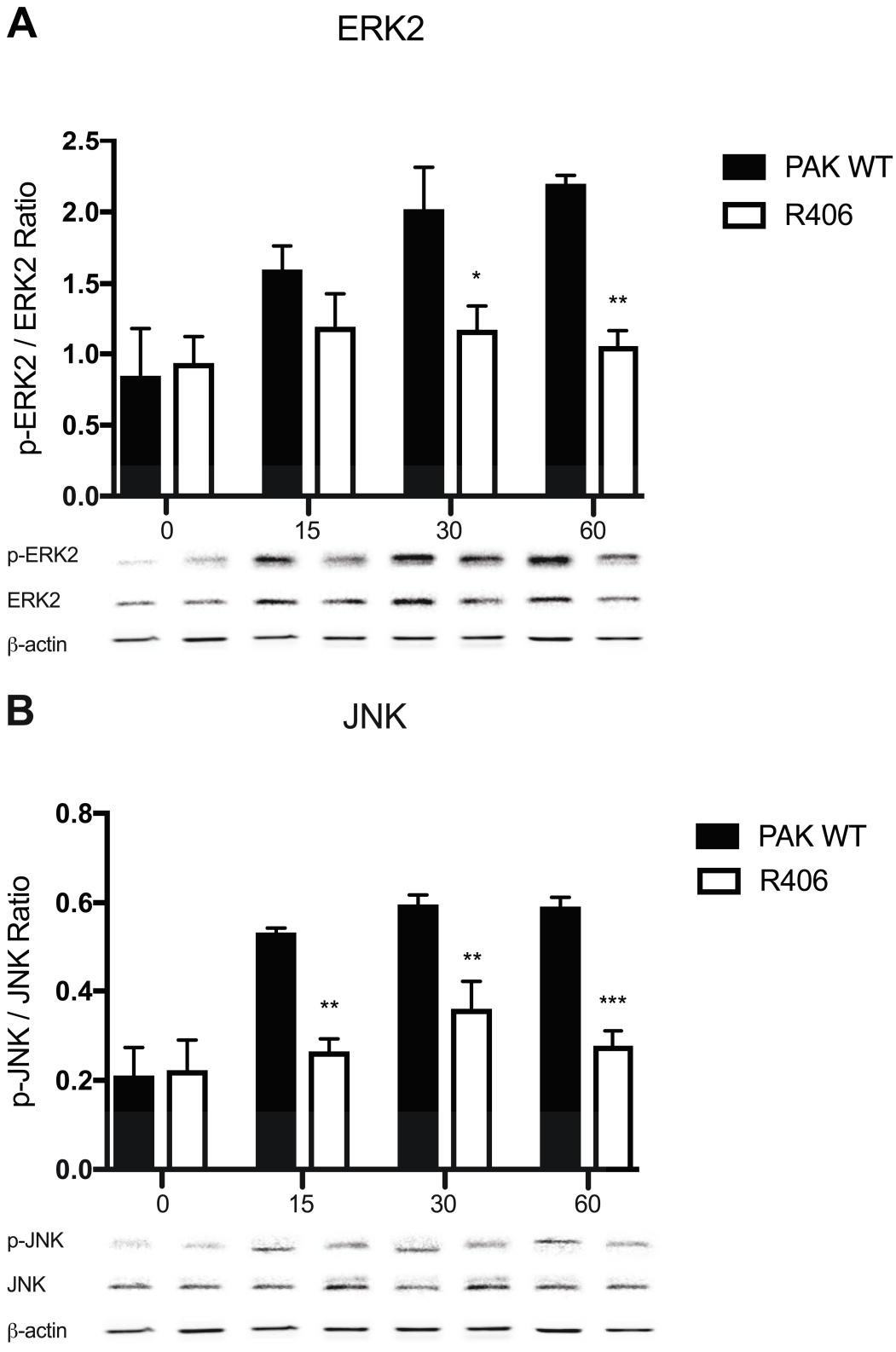
Figure 3


A
ERK2
p-ERK2 / ERK2 Ratio
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PAK WT
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PAK WT
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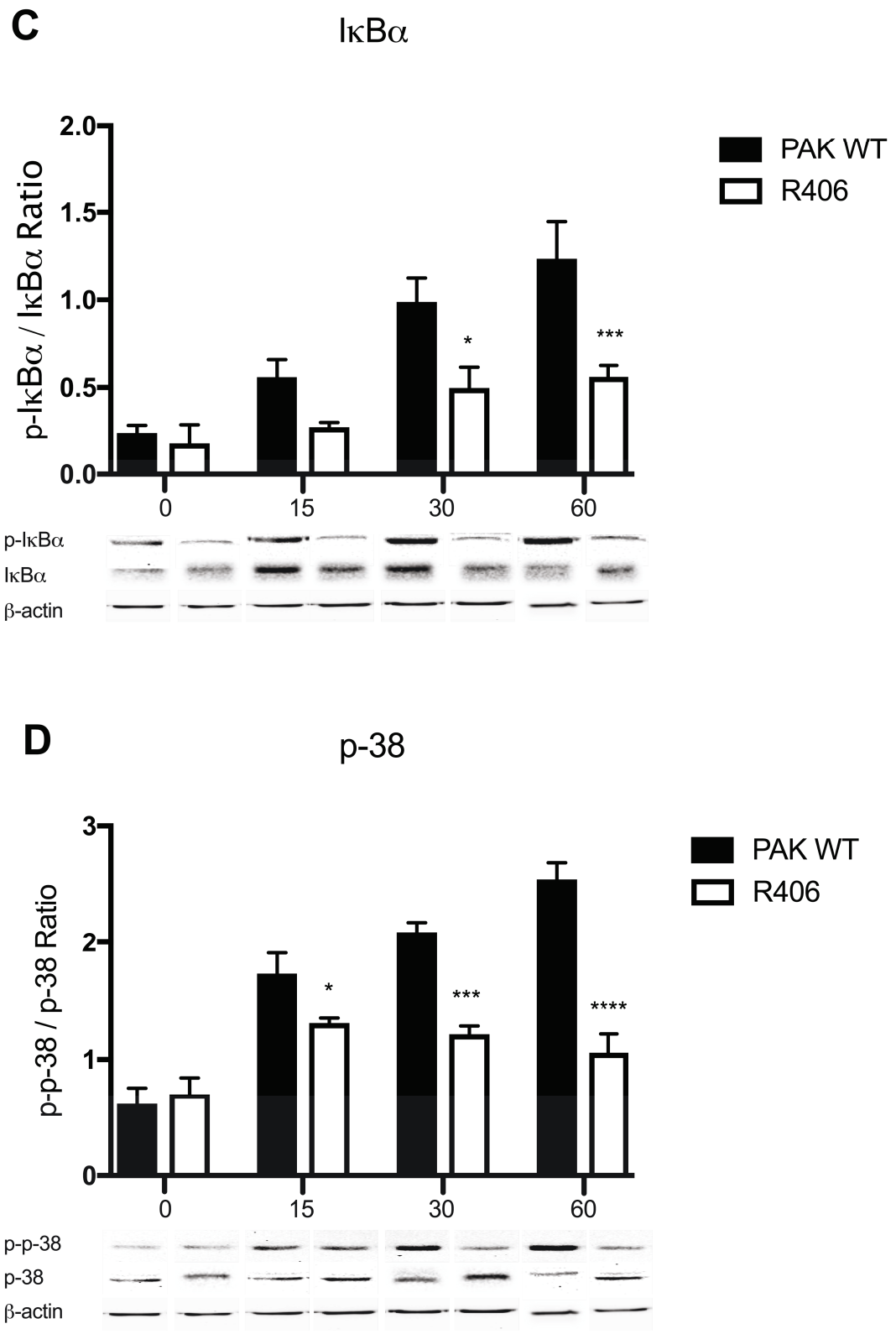
β-actin

Continue Figure 3



Chapter IV: General Discussion and Future Directions

A decade ago, a fundamental discovery that activation of NLRs results in the assembles of inflammasomes has united immunologists and microbiologists who study basic mechanisms of PRR signaling and their interaction with bacterial pathogens in infectious models with clinicians and geneticists addressing the pathogenesis of inflammatory diseases. Developments in the past few years showed how NLRs and inflammasomes are activated to promote cytokine production and cell death responses in the context of microbial infection [1]. Other findings clarified how different NLRs respond to both PAMPs and DAMPs [2]. In addition, intricate communication between PRRs, e.g., TLRs and NLRs, and microbial infection has emerged as a critical mechanism controlling immunity, and several fine-tuning mechanisms modulating PRR-induced immune responses have been discovered.

Inflammasome, i.e. high-molecular weight signaling platforms, is required for the activation of caspase-1 followed by pro-IL-1β processing into the biological active IL-1β molecule, which is a key mediator of innate immune and inflammatory responses [3]. However, there are currently several challenges facing understanding of signaling mechanisms. First, specific molecular mechanisms of NLR activation remain largely undefined and it is unclear how *P. aeruginosa*-associated molecules are sensed by NLRs and engage innate immune responses. Over the course of an *in vivo* chronic infection, *P. aeruginosa* is failing to be eradicated from the CF lung and the disease course is characterized by continuing escalation of inflammation and deterioration of lung function (Figure 1), as well as, accumulate multiple loss-of function mutations, e.g., loss of motility and decrease of expression of T3SS, proteases, pyocyanin, pyoverdin, and the

quorum sensing system [4]. Second, it is unknown whether *P. aeruginosa* strains, which have mutated during the process of adaptation in the host, alter their abilities to activate inflammasomes and can consequently evade immune responses [5]. Then in a consecutive step, previous studies found an increased level of IL-1β in BALF from CF patients infected with *P. aeruginosa* [6] that might be involved in the pathogenesis of lung disease severity in CF patients infected with *P. aeruginosa*. Studies showed that non-classical IL-1β activation is pathogen-dependent but caspase-1-independent [7]. Again, it is unknown if *P. aeruginosa* infection able to activate IL-1β by caspase-1-independent pathway. Other challenges include, studies support the role of caspase-1 to regulate several different pathways that lead to pore formation, autophagy, induction of NF-κB, cholesterol and fatty acid biogenesis, and cleaves several enzymes required for glycolysis [1]. Moreover, pyroptosis induced by *P. aeruginosa* has yet to be addressed [8]. Finally, SYK has been identified as a key mediator of NLRP3 inflammasome activation and IL-1β secretion in macrophages stimulated with fungi and crystals [9]. However, it is unknown if SYK mediates inflammasome formation and activation in response to *P. aeruginosa* infection. In brief, there are many unsolved mysteries in NLR biology with *P. aeruginosa* infection.

All of the work presented in this thesis approaches some of these challenges in addressing the overall involvement of NLRs in innate immune recognition of *P. aeruginosa*, through demonstrating either NLR-mediated caspase-1 or *P. aeruginosa*-induced IL-1β secretion. There were several reasons for this choice, to determine which *P. aeruginosa* virulence factors are essential for the activation of NLR-mediated innate immune responses, to clarify the critical role of caspase-1 following the recognition of *P.*

aeruginoa by NLR(s), and to examine, which signaling pathways are involved in NLR-mediated innate immune responses to *P. aeruginosa*. These attributes lend to clarify the role of specific bacterial virulence factors in the activation of innate immune responses to *P. aeruginosa* and will contribute to the understanding of dynamical host-pathogen interactions during the infectious process. Also, these attributes assist to identify possible pathways of persistence of *P. aeruginosa* infection during an *in vivo* infectious process, as well as clarify the mechanisms behind the involvement of SYK-mediated signaling in the regulation of innate immune responses to *P. aeruginosa* infection. Nonetheless, this work is limited by the scope of our model. First and foremost, interpreting data from an *in vitro* model need cautious as an *in vivo* there are many more cell types involved in the pathophysiology of lung inflammation and bacterial infection.

The majority of the body of work provided in this thesis was done with THP-1 cell line, monocytic and macrophage (differentiated) cells. There are several reasons for choosing THP-1 cell line as an *in vitro* model to study immune responses, which has been reviewed elsewhere [10]. Monocytes play a critical role in orchestrating of both innate defense and inflammatory responses. These cells are circulating in the bloodstream and act as gatekeepers in innate immunity. While they can differentiate into macrophages and DCs, monocytes themselves respond to various inflammatory stimuli by producing proinflammatory responses. Therefore, monocytes not only contribute to host defense against pathogenic bacteria, but are also closely associated with the pathogenesis of chronic sterile inflammation [11]. PMA, as a differentiation agent, is the most effective to obtain macrophage-like phenotype with similarities to peripheral blood mononuclear cell (PBMC) monocyte-derived macrophages based on the literature and our experience.

However, a number of factors should be taken into account i.e., the minimal concentration of PMA, incubation period with PMA, and the lenghth of resting incubation after removing PMA.

First, all of the work presented in my first study was done to determine which *P. aeruginosa* virulence factors are essential for the activation of NLR-mediated innate immune responses in THP-1 cells. That is, to identify NLRs activation and inflammasome assembling through caspase-1 activation. With this work I was able to differentiate human monocytic cells to macrophage-like cells and optimize the infectious model with a highly virulent reference strain PAK WT during different times (1-18 hrs) at various MOIs (1-10). The results were compared to the effect of *Escherichia coli* LPS, which has a strong ability to induce innate immune responses, and served as a reference for data interpretation [5,12]. As a positive control of NLR activation, stimulation with ATP has been used [9]. To clarify the role of NLR activation in innate immune recognition, *P. aeruginosa* strains with distinct expression of major virulence factors were used. To examine whether bacteria, which have mutated in the process of adaptation to the host, alter their ability to activate inflammasomes, we used a collection of *P. aeruginosa* strains that have been genetically and phenotypically characterized by other studies [13]. These strains have been isolated at different times from the beginning of an *in vivo* infection: 13 of them caused short-term infection, whereas 14 persisted in the lung over 15 years. Based on previous studies by our group [13], we hypothesized that persistent strains that have lost major virulence factors in the process of adaptation to host defense are able to escape recognition by NLRs and have a reduced ability to activate

caspase-1. As a result of the immune evasion, such bacteria become successful in the establishment of long-term parasitic interactions with the host.

In biotechnological applications, the efficiency of enzyme, which accelerates the rate of chemical reactions, for proteolysis is critical. In general, non-controlled proteolytic activity is associated with many diseases [14]. Our results showed that procaspase-1 was expressed in THP-1 cells; however, following cell stimulation and inflammasome assembling, the enzyme autoproteolysis into p10 and p20 subunits, which both is represented as an active form. Therefore, we compared between different techniques, i.e., flow cytometry and ELISA, to determine the level of active caspase-1 in both monocytic cells and macrophages. Moreover, our results with caspase-1 inhibitor indicated the successful inhibition of caspase-1 and its end product, which is biologically active IL-1β. Several methods are illustrated for the assessment of NLRs activation and inflammasome assembling, which is leading to production and secretion of caspase-1 and IL-1β (Figure 2).

Future study represents a foundation to clarify, which critical NLR-mediated innate immune mechanisms are activated following the recognition of *P. aeruginosa*. This includes looking at antimicrobial peptide human beta-defensin-2 (hBD-2). hBD-2 is essential in host defense as it causes direct lysis of Gram-negative bacteria and also activates adaptive immunity by recruiting dendritic and T cells to the site of microbial invasion [15]. It has been established that *P. aeruginosa* infection of epithelial cells induces hBD-2 gene expression and that flagella play an important role in this process [16,17]. Previous studies by our group showed that PAK WT rapidly induced hBD-2 gene expression in A549 cells [18]. However, the role of specific virulence factors in the

regulation of hBD-2 expression is poorly defined and it is unknown whether their loss in the process of *P. aeruginosa* adaptation to the host can decrease hBD2 expression. Although it has been demonstrated that activation of NOD2 leads to the induction of hBD-2 [19], the role of other members of NLR family, as well as caspase-1 have not been defined. To elucidate whether the NLR-mediated responses result in the production of hBD-2, gene silencing with small interfering (si) RNA for NLR, as well as caspase-1 inhibitor could be used.

Understanding of molecular mechanisms involved in innate immune recognition of bacterial pathogens has fundamental biological significance. Investigation of the role of NLRs in innate immune responses to *P. aeruginosa* brought new insights into the molecular pathogenesis of bacterial infections. Further studies are needed to clarify a number of potential specific NLRs that might be involved in *P. aeruginosa* infection such as: NOD1/2 [1], AIM2 [20], NLRC3 [21], NLRC4 [1], , NLRC5 [22,23], NLRP3 [24], and NLRP10 [25,26]. The findings in such study can be important for developing of novel ways to control the bacterial-host interactions and may have broader application for innate immune responses to other microorganisms.

In my second study, we used our previously established techniques to address the role of SYK kinase in the regulation of inflammasome activation by *P. aeruginosa* infection of human cells. We hypothesized that SYK mediates inflammasome activation and promotes an enhanced production of pro-inflammatory mediators by infected cells. SYK in both monocytic and macrophage cells was inactivated using the inhibitor R406 and then cells were infected with *P. aeruginosa*. The release of biologically active IL-1β was used as indicator of inflammasome activation and we found that this parameter

decreased in cells with inactivated SYK. To confirm the effectiveness of SYK inhibition, we assessed SYK phosphorylation using immunoprecipitation and Western blot. Moreover, we evaluated the effect of SYK inhibitor in airway epithelial cells, which are a major target for potential therapeutic intervention. These experiments clarified the mechanisms behind the involvement of SYK-mediated signaling in the regulation of innate immune responses to *P. aeruginosa* infection. Indeed, we have recently found that SYK inhibitor down-regulated inflammation in an *in vitro* model of *P. aeruginosa* infection [27]. An important question left unaddressed is the direct effect of SYK inhibitor on bactericidal activity of THP-1 cells. An interesting way to measure this with respect to our model would be to determine the effect of SYK inhibitor on phagocytosis of *P. aeruginosa* into infected THP-1 macrophage cells. As phagocytosis of *P. aeruginosa* with alveolar epithelial cells determined in previous studies by our group [13].

Future work is required to address the role of ROS in the mechanisms of inflammasome activation by *P. aeruginosa*. In previous studies by our group, an antioxidant N-Acetylcysteine (NAC) abrogated ROS production in A549 cells infected with PAK WT [18]. Also, the antioxidant treatment caused down-regulation of *P. aeruginosa*-induced inflammatory responses and apoptosis [18], suggesting that ROS may induce inflammasome activation in *P. aeruginosa* infection. To determine whether ROS induced by *P. aeruginosa* are essential for inflammasome activation, THP-1 cells will be pretreated with the antioxidant NAC according to previously optimized conditions [18] and then infected by PAK WT, which induces high levels of intracellular ROS [13,28]. Intracellular ROS will be detected by flow cytometry (the CM-H_2DCFDA assay) [18]. To detect inflammasome activation, we will study the release of active caspase-1

and of biologically active IL-1β. To test an alternative hypothesis that ROS directly contribute to NF-κB activation, we will concurrently study the effect of NAC on the release of inflammasome-independent cytokine TNF-α using ELISA [9]. The proposed experiments will clarify whether *P. aeruginosa*-induced inflammasome activation requires ROS production.

In conclusion, there are still many avenues of study needed to expand the ideas presented in this thesis. Remarkably, our findings suggest that *P. aeruginosa*, which lost certain virulence factors during pulmonary infection, may fail to induce caspase-1 activation and secretion of IL-1β in the process of host-pathogen interactions. This may reveal novel mechanism of the pathogen adaptation to avoid detection by NLR(s). Most importantly, the results suggest that SYK is involved in the regulation of inflammatory responses to *P. aeruginosa*, and SYK inhibitor may potentially be useful in dampening the damage caused by severe inflammation associated with this infection.

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Figure legend

Figure 1 Effect of altred CFTR function in CF lung and promotion of chronic pulmonary infection.

Figure 2 Schematic overview of appropriate techniques to evaluate production and secretion of caspase-1, IL-1β, and IL-18.

(1) Gene expression analysis of IL-1β and IL-18 by quantitative PCR. (2) Measurement of protein levels of pro-IL-1β and pro-IL-18 by ELISA technique. (2, 3, and 4) Detection of pro-IL-1β, pro-IL-18, procaspase-1, and active caspase-1 by Western blot. (4) Intracellular activity assay of active caspase-1 by flow cytometry. (5) Measurement of protein levels of active caspase-1, IL-1β, and IL-18 by ELISA technique or detection by Western blot. (1, 2, 3, and 4): following cell lysis; (5): in cell culture supernantant; PRR: pattern recognition receptors; ATP: adenosine triphosphate; ASC: apoptosis-associated Speck-like protein containing a CARD.

Figure

Figure 1

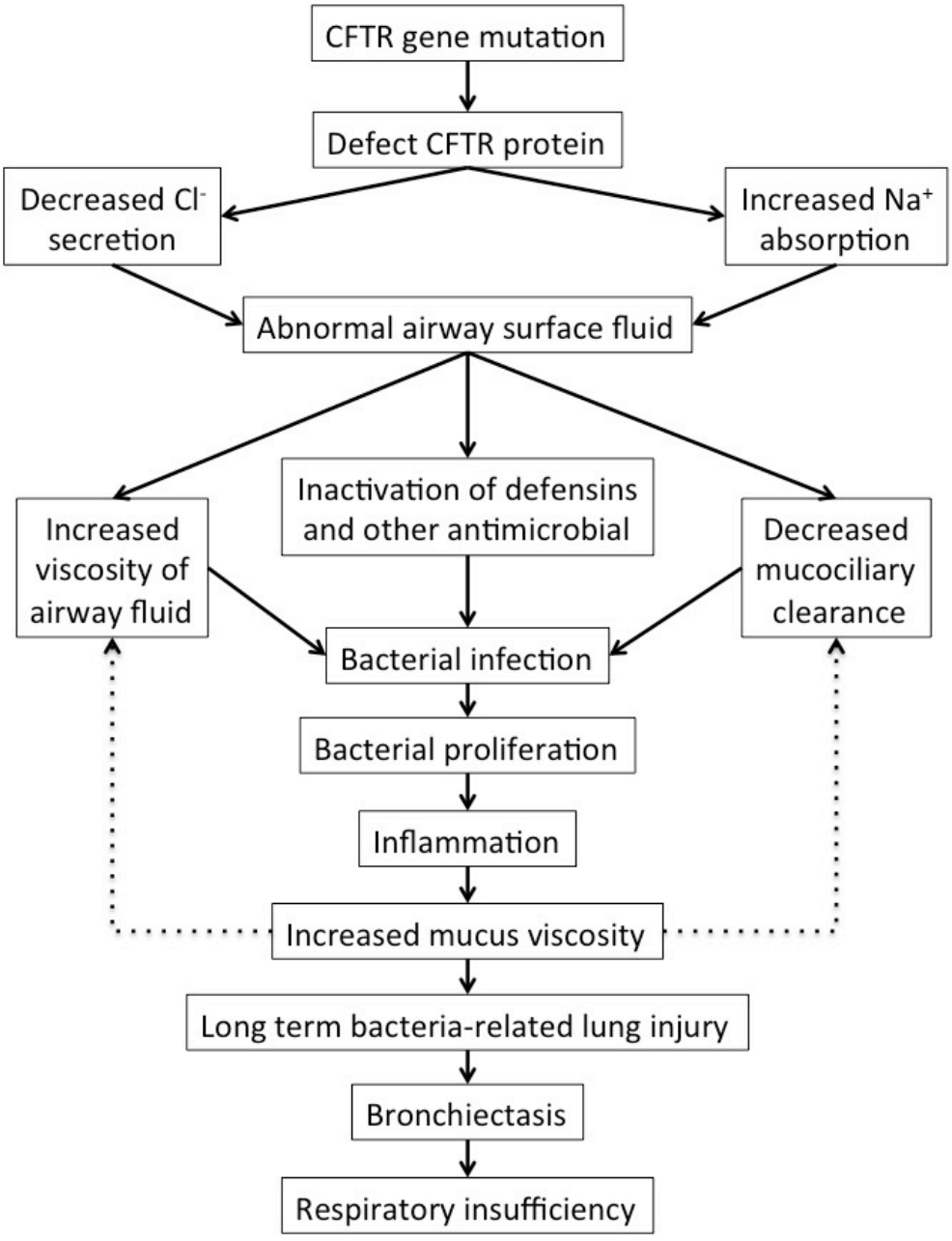
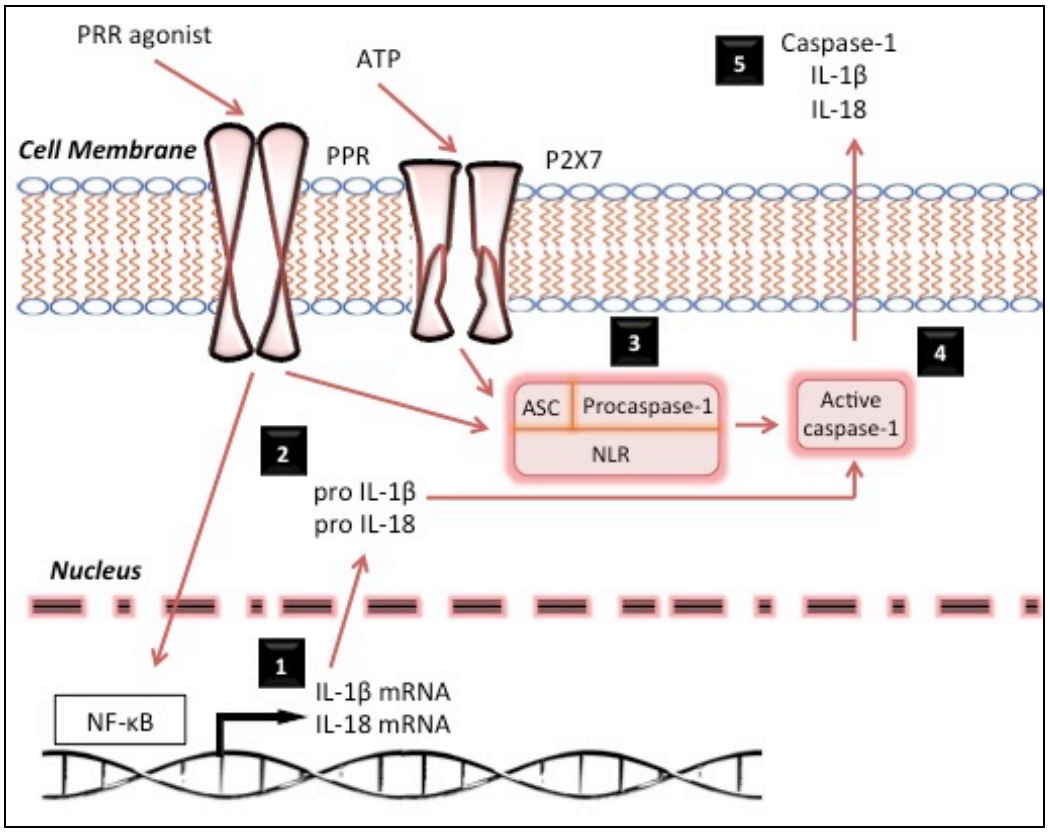


Figure 2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ATP
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Caspase-1
IL-1β
IL-18
Cell Membrane
PPR
P2X7
3
4
ASC
Procaspase-1
NLR
Active
caspase-1
2
pro IL-1β
pro IL-18
Nucleus
1
IL-1β mRNA
IL-18 mRNA
NF-κB

Publication List

Paper Published:

1. **Alhazmi, A.** (2015). *Pseudomonas aeruginosa* – Pathogenesis and Pathogenic Mechanisms. *Int J Biol*, 7(2), p44. http://dx.doi.org/10.5539/ijb.v7n2p44.
2. **Alhazmi, A.** NOD-like Receptor(s) and host immune responses with *Pseudomonas aeruginosa* Infection. Inflamm. Res. (2018). https://doi.org/10.1007/s00011-018-1132-0
3. **Alhazmi, A.**, Choi, J., & Ulanova, M. (2017). Syk inhibitor R406 down-regulates inflammation in an in vitro model of Pseudomonas aeruginosa infection. Can J Physiol Pharmacol. doi:10.1139/cjpp-2017-0307

Paper submitted:

1. **Alhazmi, A.** SYK Tyrosine Kinase as Target Therapy for *Pseudomonas aeruginosa* Infection. Journal of Innate Immunity.
2. **Alhazmi, A.** Ulanova M., *Pseudomonas aeruginosa* Infection of Human Monocytic cells Results in Caspase-1 Activation and IL-1β. Submitted to FEMS Pathogen and Diseases.